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Avian Riboflavin Binding Protein

by

Ian MacLachlan ©

A Thesis
Submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

Fall, 1994

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To my friends and family.

ABSTRACT

Riboflavin binding protein (ribBP) is an essential component of chicken eggs: it supplies the oocyte with sufficient amounts of the vitamin riboflavin to sustain embryonic development until hatching. I have begun to investigate the mechanisms underlying ribBP transport pathways by molecular characterization of a relevant mutation in chicken. The autosomal recessive *rd* allele prevents the synthesis of functional ribBP. cDNAs for ribBP from normal (*Rd)* and deficient (*rd*) animals were cloned and sequenced. The *rd* allele was associated with a 100-nucleotide deletion in the messenger RNA for ribBP. Genomic cloning demonstrated that the deletion corresponds precisely to an exon. The splice site following this exon contains a G to A mutation at position 1 of the downstream 5′ splice donor sequence. The effect of this anomaly and the cause of the *rd* phenotype is the loss of the 100 bp exon during the splicing process. Other rare splicing products resulting from the *rd* mutation include those arising from cryptic splice site activation, skipping of two exons and normal splicing in spite of the 5' G to A mutation.

Serum ribBP (sribBP) has some binding affinity for the oocyte-specific 95-kDa lipoprotein receptor. However, in serum, ribBP associates with the yolk precursor vitellogenin (VTG). In the presence of VTG, ^{125}I-labeled sribBP binds to the 95-kDa receptor, and under certain conditions also to the avian oocyte low density lipoprotein receptor-related protein. The interaction between ribBP and the 95-kDa receptor and VTG requires Ca^{2+} and P_i. Serum, yolk and egg white ribBPs are all capable of VTG-associated receptor binding demonstrating that (i) the carboxyterminal 11 or 13 amino acids removed from sribBP upon oocytic uptake are not involved in receptor binding, and (ii) receptor binding and/or association of ribBP with VTG is not dependent on the carbohydrate structure

present on sribBP. The results indicate that the oocytic uptake of sribBP is mediated, through association with VTG, by the 95-kDa receptor and possibly other oocytic members of the low density lipoprotein receptor gene family, adding an interesting and novel variation to ligand recognition by these receptors.

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Dr. Harold B. White III. provided me with the cDNA clone for riboflavin binding protein as well as invaluable samples of *rd* serum , egg yolk and egg white. Not only were these samples necessary for the characterization of the *rd* allele, they provided a convenient genetic control for some of the experiments which characterized the uptake of riboflavin binding protein in the oocyte. Dr. White has also been a source of inspiration and encouragement.

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LIST OF ABBREVIATIONS AND SYMBOLS

apo	apolipoprotein
apo B	apolipoprotein B
apo II	apolipoprotein VLDL-II
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DEAE	diaminoethyl
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminotetraacetic acid
EGF	epidermal growth factor
FH	familial hypercholesterolemia
HDL	high density lipoprotein
IgG	immunoglobulin G
IODO-GEN	1,3,4,6-tetrachloro- 3α, 6α-diphenylglycouril
kb	kilobase
kDa	kilodalton
K_d	ligand concentration that gives half maximal binding
M_r	molecular weight
mRNA	messenger ribonucleic acid
nt	nucleotide
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
rd	riboflavin binding protein deficient allele
Rd	wild type allele
ribBP	riboflavin binding protein
R/O	restricted ovulator

s	serum
sribBP	serum riboflavin binding protein
SDS	sodium dodecyl sulfate
VLDL	very low density lipoprotein
VTG	vitellogenin
w	white
wribBP	egg white riboflavin binding protein
y	yolk
yribBP	egg yolk riboflavin binding protein

A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil

A	ala	alanine
C	cys	cysteine
D	asp	aspartate
E	glu	glutamate
F	phe	phenylalanine
G	gly	glycine
H	his	histidine
I	ile	isoleucine
K	lys	lysine
L	leu	leucine
M	met	methionine

N	asn	asparagine
P	pro	proline
Q	gln	glutamine
R	arg	arganine
S	ser	serine
T	thr	threonine
V	val	valine
W	trp	tryptophan
X		any amino acid
Y	tyr	tyrosine

CHAPTER I

INTRODUCTION

I. INTRODUCTION

The loss of the bio-synthetic capabilities required for the de-novo synthesis of vitamins such as B12, A and riboflavin was accompanied by the evolution of mechanisms for their transport. Concomitant with this development was the evolution of processes which deliver these vitamins to the embryo. Oviparous species, in particular, have to sequester these nutrients in the oocyte prior to egg laying and the onset of embryonic development. The uptake mechanisms of many of the yolk precursors, including the vitamin binding proteins, have yet to be elucidated. The purpose of this study is to characterize the mechanism by which one such vitamin binding protein, riboflavin binding protein, is taken up and sequestered by the growing avian oocyte. A mutant strain of chickens, the *rd* strain, lacks a functional riboflavin binding protein. The molecular nature of the defect associated with this allele is elucidated here in the hope that it may tell us more about the biochemistry of riboflavin binding protein.

This study was initiated in the laboratory of Dr. Wolfgang Schneider, and its success was precipitated by several factors. Firstly, the laboratory as a whole was devoted to the study of yolk precursor uptake. The uptake mechanisms of two non-vitamin avian yolk precursors have been characterized in our laboratory (1, 2). The compounds very low density lipoprotein and vitellogenin are bound by a specific oocyte membrane receptor and taken up in the growing oocyte via receptor mediated endocytosis. As a result of these studies several techniques had been developed which enabled one to biochemically visualize receptor-ligand interactions. Secondly, although riboflavin binding protein is one of the minor yolk precursors, it had been the subject of intense study by a number of groups. As such the avian riboflavin binding proteins were the best characterized of all the riboflavin binding proteins. Thirdly, at the time this study was begun, the cDNA for egg white riboflavin binding protein had just been cloned. This greatly expedited the elucidation of the *rd* defect.

The following section is intended to provide the reader with the information required to understand the context and implications of the research contained in this thesis. It begins with a brief discussion of the process by which an oocyte grows in the laying hen. Also included is a discussion of the yolk precursors including riboflavin binding protein. There is a discussion of the current understanding of the lipoprotein receptor family which should put the results of the binding studies into context. Finally, to help the reader understand the implications of the molecular studies, RNA splicing is introduced.

I. A. OOCYTE GROWTH

I. A. 1. OOGENESIS

Oogenesis is the process by which primary oocytes are formed during embryonic development. Early in avian embryonic development the right ovary and oviduct are reabsorbed. Consequently, most avian species have only one functional branch of the usually bi-symmetrical reproductive system(3). Oocytes are derived from oogonia. Oogonia are in turn derived from differentiated primordial germ cells(4). Oogonia proliferate mitotically in the left ovary until signalled to enter meiosis. Once they enter meiosis they advance to the prophase stage of division I where they become arrested. It is these cells, arrested in prophase, which are called primary oocytes. Prophase of division I is characterized by two important features. First, the decondensation of the chromosomes forming the looped "lampbrush" configuration which is indicative of and may facilitate RNA synthesis.

Second, duplicate sets of diploid chromosomes result from arrested meiosis. This provides the cells with double the normal amount of DNA available for RNA synthesis. Primary oocytes must accumulate all the material that will later direct early embryonic development. This includes ribosomes, mitochondria, tRNAs, mRNAs, and yolk precursors. The process by which yolk precursors are taken up is termed vitellogenesis.

I. A. 2. VITELLOGENESIS

Vitellogenesis in avian species is characterized by three phases(5). The formation of the primary oocyte is followed by an initial slow growth phase which lasts for months or years. During this period very little yolk is deposited. This senescent phase is followed by a period of slow yolk deposition. This second slow growth phase is thought to last some 60 days and results in an increase in the mass of the oocyte to approximately 0.5 g. The third and final growth phase lasts only seven days. It is during this phase that the vast majority of the yolk is laid down. The growth rate is so rapid that during these last seven days as much as 1.0 - 1.5 grams of protein may be taken up into the oocyte each day, increasing its weight from an initial 0.5 to a final 19 g. This dramatic increase is due in part to the specific uptake of many of the yolk precursors. VLDL and VTG, the major yolk precursors in avian species, are taken up by a specific receptor localized on the oocyte plasma membrane(1, 2, 6). Many other yolk precursors are concentrated in the oocyte including ribBP(7), retinol binding protein (8), transferrin (9), transthyrethrin (8) and immunoglobulins (10). RibBP, present in the serum at 0.064 mg/ml is concentrated roughly six-fold in the egg yolk at 0.56 mg/ml (11). This is comparable to the relative increase in VTG concentration in the egg yolk which is roughly six-fold that of serum. It is interesting that many of the minor yolk precursors are synthesized hepatically and enter the bloodstream where they bind vitamins and other important metabolic cofactors. Some of these proteins appear to have no role other than to transport these cofactors to the growing oocyte. The mechanisms by which these precursors are concentrated in the yolk are only now being elucidated.

Following the final rapid growth phase the oocyte is released from the ovary via ovulation. Ovulation is the process by which the follicle containing the mature primary oocyte bursts releasing the secondary oocyte into the oviduct. However, in order to properly understand the process of vitellogenesis it is necessary to firstly understand some aspects of follicular morphology.

I. A. 3. FOLLICULAR MORPHOLOGY

The primary oocyte lies within a complex multilayered structure named the follicle (Fig. I. 1.). The follicle has three known roles. First, it maintains the structural integrity of the growing oocyte. Second, it provides the cellular signals required for oocyte maturation and ovulation. Third, it provides the oocyte with continuous access to yolk precursors. RibBP and other yolk precursors enter the follicle via the heavily vascularized theca cell layer which surrounds the oocyte. The theca cells are a layer of fibroblast-like cells embedded in collagenous fibers. Inside this layer is a collagenous basement membrane, also called the basal lamina, which separates the theca cells from the granulosa cells. In the preovulatory follicle the granulosa cell layer is one cell thick. The surface of the cells contacting the basal lamina is smooth, while the surface exposed to the oocyte plasma membrane is heavily invaginated and coated with microvilli(12). Granulosa cells play a role in follicle hormone production and are known to secrete progesterone(3). Some of the minor yolk precursors may be provided directly to the oocyte by the granulosa cells. Separating the granulosa cells from the oocyte is the perivitelline layer. Yolk precursors entering the follicle from the circulation must move through the theca cell layer, cross the basal lamina and pass between the granulosa cells before coming into the perivitelline layer and eventually contacting the oocyte plasma membrane. Clustered on the membrane surface in clathrin coated pits are specific receptors for VLDL and VTG and possibly other yolk precursors. VLDL and VTG are taken up into the oocyte specifically against an increasing concentration gradient.

When an oocyte matures, it is released from the follicle via rupture along a site called the stigma. This is a band of poorly vascularized tissue which tears open during ovulation.

I. A. 4. OOCYTE MATURATION AND OVULATION

Ovulation is the process by which the follicle ruptures and releases the mature oocyte into the opening of the oviduct, the infundibulum (Fig. I. 2.). However, if an oocyte is to reach its full potential two things must happen first. As well as being physically released from the follicle the oocyte must also be released from arrest in division I of meiosis so that it may enter metaphase of division II in preparation for fertilization. This temporary resumption of meiosis is best understood in the *Xenopus* model. In amphibia it is thought to be triggered by the release of progesterone from the granulosa cells(13). Progesterone interacts with oocyte cell surface receptors which in turn results in a decrease in intracellular levels of cyclic AMP and diacylglycerol(14). This results in inactivation of serine/threonine protein kinases and the expression of cyclins, proteins involved in the regulation of the cell cycle. Cyclin interacts with another protein, maturation-promoting factor, in an autocatalytic cascade that leads to the phosphorylation of nuclear lamins and chromosomal proteins. The result is a disruption of the nuclear envelope and condensation of chromatin bringing the cell to metaphase. Similar systems are thought to regulate this process in other animals.

I. B. THE YOLK PRECURSORS

The laid egg must contain an exquisite balance of nutrients which will be used to fuel the growth and development of the embryo. The majority of these nutrients are contained in the egg yolk. The two major yolk precursors, VLDL and vitellogenin (VTG), provide the embryo with sources of triglycerides, phospholipids, cholesterol, amino acids, calcium and phosphate. Their uptake is mediated by a specific cell surface receptor, present on the oocyte, which specifically binds both of these components (6). The minor yolk precursors, such as the vitamin binding proteins riboflavin binding protein and retinol binding protein are equally requisite for embryonic development, yet the mechanisms by which these proteins are taken up into the oocyte remain to be determined.

The term *yolk precursors* is often used to describe nutrients destined for yolk deposition. This phrase refers to the fact that many yolk components are modified following uptake and hence the precursors differ from the forms found in the yolk of the mature oocyte. This modification often involves proteolytic processing. As we shall see this is a theme common to many of the yolk components.

I. B. 1. VLDL

Lipoproteins are specific aggregates of lipids and proteins. They serve as a transport vehicle for water insoluble lipids, shuttling the lipids from the site of synthesis or dietary uptake through the circulation to a site of absorbtion where the lipids are either stored or degraded. Perturbations in lipoprotein metabolism in man have been shown to cause various pathogenic states. Conditions which involve an increase in serum levels of very low density lipoproteins often result in atherosclerosis, a major cause of premature death in developed countries (15, 16, 17). For this reason lipoprotein metabolism has been the focus of a great deal of research.

Lipoproteins are divided into classes based on their buoyant density (18). These class distinctions have a physiological relevance, since the density of the lipoprotein particle is related to the lipid composition and structure. Very low density lipoproteins (VLDL) are lipoproteins which fractionate with a buoyant density lower than 1.006 g/ml. Their very low density is due to their large size and triglyceride rich core. In mammals another very low density particle exists. Chylomicrons are large (100-250 nm) triacylglycerol-rich particles which are the major carrier of dietary lipids. They are synthesised in the intestine and secreted into the lymphatic system. After entering the jugular vein via the thoracic duct they travel through the circulation where they are acted on by the enzyme lipoprotein lipase. Lipoprotein lipase is located on the vascular epithelium where it hydrolizes trigycerides on the surface of the chylomicron particle. The resulting particle, a chylomicron remnant, has a much smaller size and increased density due to the loss of triglycerides. Chylomicron remnants are taken up rapidly in the liver by a putative chylomicron remnant receptor. Chylomicrons are known to bind to members of the lipoprotein receptor family by virtue of the apolipoprotein apo E and it has been proposed that one or more of these lipoprotein receptors may function as a remnant receptor. Avian species differ from mammals in that they synthesize neither chylomicrons nor apo E (19). Transport of dietary lipids in avian species is accomplished by large, intestinally synthesized VLDL-like particles called portomicrons. These particles enter the circulation via the portal vein and are thought to be cleared extremely rapidly. As such they are poorly characterized and little is known about their apolipoprotein content. Avian VLDL differs significantly from its mammalian counterpart. Human VLDL contains the apolipoproteins apo B, apo CI, apo CII, apo CIII and apo E (18). Avian VLDL contains the avian apo B homologue. In laying hen VLDL an additional apolipoprotein is present. This apolipoprotein, apolipoprotein apo-VLDL II or apo II, is synthesised in response to estrogen and as such is not present in rooster VLDL (19, 20). Roosters also differ from laying hens in that the major lipoprotein species is HDL rather than VLDL.

I. B. 1. a. APOLIPOPROTEIN B

The receptor-binding characteristics of VLDLs are conferred by apo B. Avian apo B, however, has only been partially characterized. It has a molecular weight of approximately 512 kDa. The carboxyterminal 10% of the amino acid sequence has been determined but surprisingly it shows a rather low (31% at the level of the corresponding nucleotide sequence) homology to human apo B (21). In mammals, the synthesis of apo B is relatively insensitive to estrogen. However, in avian species, apo B synthesis increases in response to estrogen. Exposure to estrogen activates apo B transcription. 24 hours after exposure to estrogen apo B mRNA levels peak at 2,500 copies per cell (21).

Human apo B has been studied in great detail and several of the findings may be relevant to the avian system. The 14 kb cDNA for human apo B codes for a 4563 amino acid protein containing a 27 residue signal peptide (22). Apo B is extensively glycosylated at 13 N-linked sites(23). Avian apo B is also known to be N-linked glycosylated. The large size and lipophilic nature of apo B has inhibited the elucidation of its gross structural features. However, sequence analysis predicts that apo B is 43 % α helix, 21% β sheet, 16 % β turn and 20 % random structures(23). These data correlate well with data from circular dichroism studies. A common structural feature of many apolipoproteins is the presence of amphipathic helices. An amphipathic structure is a structure which forms with hydrophobic residues arranged on one side and hydrophilic residues on the other. Amphipathic helices are thought to be involved in the association and intercalation of apolipoproteins within the lipid bilayer. Some apoproteins can exchange from one lipoprotein particle to another by virtue of these regions. Apo B does not exchange between lipoproteins. It is anchored in the VLDL particle by amphipathic β–sheets(18). It is thought that receptor recognition may involve charged portions of these amphipathic structures. Various strategies have been employed in attempts to map the receptor recognition site (or sites) in apo B. Thrombolytic cleavage of human apo B results in three apo B fragments, T1, T2 and T3. When these fragments are purified and reconstituted with canine HDL_C, a subfraction of HDL, which had been previously treated to eliminate receptor binding activity, all three of the reconstituted fragments were able to bind the receptor (24). This would seem to favor a multiple site model where receptor recognition was not confined to one region of the molecule. However, the amino terminal 48% of apo B is not thought to be involved in receptor recognition. The reason for this is because apo B-48, a naturally occurring truncated form of apo B produced normally in the mammalian intestine, does not bind to the LDL receptor (25, 26). In support of this evidence, a study of several monoclonal antibodies directed against apo B identified one which was able to inhibit apo B binding to the human LDL receptor (27). The recognition site for this antibody was mapped to a region near the T2/T3 cleavage site in the carboxyterminal portion of the protein. Near this region are two clusters of positively charged amino acids. It is thought that these regions may be brought together by the formation of a disulfide bond and that this may constitute the receptor binding domain.

However the results in this area are unclear. Currently, investigations are underway whereby the gene coding for human apo B is being expressed in transgenic mice in an effort to further elucidate the requirements for receptor binding. It is reasonable to assume that, due to the high level of conservation between avian and mammalian lipoprotein receptors (see later section), that similar mechanisms are involved in the receptor recognition of avian apo B.

I. B. 1. b. APOLIPOPROTEIN VLDL-II

In addition to apo B, VLDL in the laying hen contains a second apolipoprotein, apo VLDL-II or apo II. Both the cDNA and gene for apo II have been cloned(28, 29). Apo II is a 82 amino acid protein after the removal of a 24 residue signal peptide. It dimerizes via the formation of one intermolecular disulfide bond. Apo II clearly lacks any kind of receptor binding activity. It does however have an essential role in avian lipoprotein metabolism (30). In mammals, nascent VLDL begins a series of changes as soon as it is released into the plasma. These changes include the rapid catabolism of VLDL triglycerides by the enzyme lipoprotein lipase (LPL). In the laying hen the VLDL is targeted for deposition in the oocyte and must be protected from degradation. Apo II specifically inhibits LPL protecting the VLDL particle until it reaches the oocyte. The mechanism of this inhibition is not well understood. Recently, it was observed that Japanese quail VLDL contains an apo II which appears as a monomer when analyzed by SDS polyacrylamide gel electrophoresis. Amino acid compositional analysis revealed that the Japanese quail apo II lacks the cysteine required for dimerization. In order to better understand the mode of LPL inhibition I have cloned the cDNA for Japanese quail apo II. There are several structural differences between the chicken and quail apo II. The translation of the cloned cDNA sequence confirms that quail apo II lacks the cysteine required for dimerization and shows that the carboxyterminal portion of the molecule is poorly conserved. Preliminary data suggest that in spite of these structural differences, quail apo II also inhibits LPL. Given this information, it should be possible to draw some conclusions as to which structural features of apo II are important for LPL inhibition.

The synthesis of apo II is under the strict control of estrogen and as a result it is only present in laying hen VLDL (31). The gene for apo II has been the subject of extensive analysis which has identified some of the regions involved in its control (29). This control is at the level of both transcriptional regulation and mRNA stability. The 3' untranslated region is thought to contain looped structures which become evident upon secondary structural analysis. These loops contain the sequence GAUG which is thought to be the target of exonuclease attack in the absence of estrogen(32). This triggers the further degradation of the message. Interestingly, all three copies of the GAUG motif found in the chicken transcript are conserved in the cDNA sequence for Japanese quail apo II.

I. B. 2. VITELLOGENIN

Vitellogenin (VTG) is often referred to as the primordial apolipoprotein (18). VTG has been found in organisms as diverse as the primitive roundworm *Caenorhabditis elegans* , sea urchin, *Drosophila* , *Xenopus* and the chicken (33). VTG is a high density lipid carrier. Since only 20% of its weight is lipid and up to 2% of its weight is phosphate it has a density > 1.21 g/ml and upon centrifugation fractionates with non-lipoprotein serum proteins (34). Synthesized in the liver, VTG is secreted as a 440 kDa dimer. Following oocytic uptake, VTG is proteolyzed yielding lipovitellin-I, lipovitellin-II, phosvitin and small phosvitin-like peptides called phosvettes (35). Characterization of the precursor-product relationship between VTG and these proteins has been complicated by the presence of multiple copies of the gene for VTG. In fact three genes for VTG are expressed simultaneously in avian species(36). These genes VTG I, VTG II and VTG III code for distinct, yet similar proteins. The gene for VTG II, the most abundantly expressed of the vitellogenins, has been cloned(37). This gene spans 20.3 kb and codes for a 5.7 kb message. The phosvitin portion of VTG is found at amino acid residues 1112 to 1328 of the native VTG II gene product. Of these 210 amino acids, 203 are coded for by one exon. Phosvitin is an extremely serine rich protein, and with more than 100 phosphorylation sites it is the most highly phosphorylated protein known. Its role in vertebrate oogenesis is thought to be related not only to the large amounts of phosphate but also the Ca^{2+} bound by the protein. The Ca^{2+} carried by phosvitin may be involved in embryonic bone formation. Since the phosvitin coding region of the avian VTG gene shows the lowest homology to the Xenopus gene and is missing entirely in the gene for Drosophila VTG, one would be tempted to conclude that this function is a relatively recent evolutionary development (38, 39). The lipovitellins are coded for by the 5' and 3' ends of the VTG II gene. Lipovitellin-I, a 110-140 kDa protein, is derived from the amino terminus of VTG. Lipovitellin-II, a 30 kDa protein, is found at the carboxyterminus of VTG(37).

Stifani et al. showed that the receptor binding characteristics of VTG are mediated by either lipovitellin-I or lipovitellin-II but not phosvitin(40). However they were not able to determine which of the two lipovitellins was responsible for binding. Comparison of the amino acid sequences of VTG from *Xenopus* , and chicken has identified conserved structural features in these proteins. The vitellogenins contain two clusters of positively charged and hydrophobic residues which resemble a region in the putative receptor binding site of apo E (41). These regions are located in the lipovitellin-I position of VTG at residues 1079-1084 (Lys-Leu-Lys-Arg-Ile-Leu) and 493-498 (Leu-Lys-Arg-Ile-Leu-Lys) and correspond to the region in human apo E between residues 143-150 (Lys-Leu-Arg-Lys-Arg-Leu-Leu-Arg). Similar sequences are located in the positively charged regions of human apo B which have been implicated in binding to the LDL receptor(23). One such region is located between residues 3359-3368 (Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys-Leu) of apo B. These data support the hypothesis that VTG may represent an avian counterpart to apo E, and that the apolipoproteins may have coevolved with the LDL receptor family, maintaining the characteristics required for receptor binding.

I. B. 3. THE MINOR YOLK PRECURSORS

The subject of this thesis, riboflavin binding protein, is one of a group of minor yolk precursors called livetins. These soluble proteins constitute approximately 10% of the total dry weight of the yolk. They include riboflavin binding protein, retinol binding protein, biotin binding protein, transferrin, immunoglobulin Y, α_2-macroglobulins and other proteins(42). The genes for some of these proteins have been cloned. Many of them are expressed in the liver under the control of estrogen. Riboflavin binding protein will be discussed in detail in the next section.

I. C. RIBOFLAVIN BINDING PROTEINS

I. C. 1. AVIAN RIBOFLAVIN BINDING PROTEINS

Riboflavin binding protein (ribBP) is a 29 kilodalton phosphoglycoprotein, synthesized in the liver and secreted into the bloodstream of the laying hen (43). Holo-ribBP is taken up by the developing oocyte and is deposited as a complex in this giant cell. RibBP is also synthesized and secreted by the oviduct. Oviducally synthesized ribBP is incorporated in the egg white (44). Egg white riboflavin binding protein (wribBP) is the most accessible and therefor best characterized of the three forms of riboflavin binding protein. Its amino acid sequence has been determined (45) and the cDNA coding for it has been cloned (46). The gene specifies a 17 amino acid signal sequence at the amino terminus which is cleaved from the mature peptide (Fig. I. 3.). Two carboxyterminal arginines predicted by the nucleotide sequence are not seen in the mature peptide. RibBP is very stable and resists denaturation even at very high temperatures. This is due in part to the maintenance of its conformation by 9 intramolecular disulfide bonds. At least one of these is essential for the binding of riboflavin (47). Riboflavin binding protein has been crystallized (48), but production of heavy atom derivatives has proved elusive and the tertiary structure of ribBP remains unresolved (49). RibBP is post-translationally modified in ways which may have functional implications. The protein is glycosylated via two N-linked sites at asparagines 36 and 147. The removal of terminal sialic acid residues from yolk ribBP (yribBP) results in a decrease in the oocytic uptake of labeled protein *in vivo.* (50) This may imply a role for the carbohydrates in yolk deposition or may be a reflection of differential hepatic uptake. RibBP also contains eight closely spaced phosphoserine residues clustered near the carboxyterminus (Fig. I. 3.). These residues lie between glutamic acids 184 and 198 and contribute to the charge on the highly ionic carboxyterminal region of the protein. They are thought to play a role in the oocytic uptake of ribBP(51) . Dephosphorylation of purified yribBP results in a decrease in oocytic uptake of ^{125}I-labeled ribBP *in vivo*. Recently it was shown that ribBP can self-associate (52). This self-association involves cross linking mediated by calcium phosphate, presumably involving the participation of the phosphoserines. It has been hypothesized that the oocytic uptake of ribBP may involve an interaction between ribBP and VTG and that this interaction could be mediated between the clustered phosphoserine residues in RibBP and those in the phosvitin moiety of VTG(43).

Although egg white ribBP (wribBP) is the best characterized of the riboflavin binding proteins, its function is not known. Chickens are unique among avian species in that the ribBP in their egg albumin also contains some riboflavin; other birds' wribBP contains no riboflavin. RibBP in the chicken egg white is not saturated with riboflavin, in fact less than 16% of wribBP is complexed with riboflavin (11) (53).

Although all are products of the same gene (44) there are several differences between oviducal wribBP and that found in the serum or yolk. Serum ribBP (sribBP) is synthesized in the liver and has the same amino acid sequence as wribBP (45). sribBP is also glycosylated at asparagines 36 and 147, however the composition of the carbohydrates in sribBP is more complex than in wribBP. sribBP contains more sialic acid and galactose than wribBP and contains fucose while wribBP does not (7, 50). These differences reflect the sites of synthesis of the two proteins. Recently the structure of the sugar chains in yribBP were established (54); yribBP has biantennary and triantennary sialic acid containing sugar chains. These sugars are similar in structure to those of phosvitin and other hepatically synthesized yolk proteins (55) yet differ from those of oviducally synthesized wribBP (56). Analysis of the structure of sribBP showed that there is apparently no modification of the glycosylation following oocytic uptake, the sugars in yribBP and sribBP are identical (54). The differences in glycosylation between sribBP and wribBP result in a difference in molecular weight and charge. This is manifested on SDS-PAGE gels where wribBP migrates faster than sribBP. In contrast to wribBP, sribBP in the serum is saturated with riboflavin (11). The role of sribBP seems clear. Its function is to carry riboflavin into the developing oocyte. Once in the oocyte, yribBP undergoes an interesting post-endocytotic modification: specific proteolytic cleavage at leucine 206 or lysine 208 (7). Limited proteolytic cleavage of yolk proteins has been observed previously. Both VTG (57) and apo B, the protein moiety of VLDL (58) (59) are specifically degraded upon entering the oocyte. Vitellogenin is converted to lipovitellin and phosvitin, and apo B is converted into a number of defined fragments. The protease responsible for this partial hydrolysis of yolk proteins has been characterized and cloned in our laboratory and has been shown to be cathepsin D (60). The function of the proteolysis is not well understood. Yolk ribBP, lacking 11 or 13 residues at the carboxyterminus, binds 8-substituted riboflavins less tightly than does wribBP (61). Cleavage of the carboxyterminal portion of ribBP may be a device preventing the binding of internalized ribBP to receptors recycling to the cell surface, assuming such receptors exist. This seems unlikely since ^{125}I-labeled yribBP is readily taken up by growing oocytes *in vivo* (50). The removal of the carboxyterminus could be involved in the release of riboflavin by yribBP.

The requirements for the binding of riboflavin have been relatively well characterized. In the bloodstream apo-ribBP binds riboflavin derived from the diet in a 1:1 molar ratio. RibBP also binds FMN ($K_a = 7.3 \times 10^5 \text{ M}^{-1}$) and FAD ($K_a = 7 \times 10^4 \text{ M}^{-1}$) (62) although not as strongly as it binds riboflavin ($K_a = 1.3 \times 10^9 \text{ M}^{-1}$). Of the eight disulfide bonds in ribBP, only two are necessary for the binding of riboflavin (63). The binding of riboflavin is thought to occur in a pocket which contains a number of tryptophan residues which are more accessible in the apoprotein than in the holo-protein. The binding of riboflavin to apo-ribBP quenches the fluorescence of at least one tryptophan in ribBP and entirely quenches the intrinsic fluorescence of riboflavin. Circular dichroism measurements of the apo and holo proteins showed two Cotton effects which have been attributed to a strong, and importantly, stereospecific binding of riboflavin to ribBP (64). The binding of riboflavin to ribBP is relatively pH independent in the range from pH 6 to pH 9. However, below pH 6 there is a rapid increase in K_d. This pH profile may be significant in terms of the fate of internalized riboflavin. Yolk precursors are stored and processed in the lumen of endosomes, the pH of which is in the range pH 5.0 to 6.0. It is possible that following endosomal deposition, riboflavin is bound less tightly by ribBP making it more accessible to the developing embryo.

Riboflavin itself is not required for the oocytic uptake of riboflavin binding protein (65). Laying hens were fed diets containing either normal or reduced amounts of riboflavin and the serum and eggs were assayed for ribBP. When fed a diet reduced in riboflavin, laying hens continue to synthesize apo-ribBP which is taken up by the growing oocyte. Therefor the mechanism responsible for oocytic ribBP uptake must not differentiate between apo-ribBP and holo-ribBP. The amount of ribBP in the serum, yolk and egg white is independent of the amount of riboflavin in the diet (11). The mechanism responsible for the control of ribBP synthesis is not sensitive to riboflavin. RibBP synthesis is however sensitive to estrogen (66). Administration of estrogen to immature male chicks, which do not normally synthesize ribBP, results in production of ribBP which peaks at 48 hours. Repeated stimulation results in a two-fold increase in the level of ribBP produced, which indicates an estrogen memory effect. Progesterone on the other hand has no effect on ribBP synthesis. It seems that the mechanisms which control the synthesis of ribBP may be similar to those which regulate the production of VTG and apo II.

I. C. 2. AVIAN RIBOFLAVINURIA

Riboflavin, bound by ribBP, is transported to the oocyte and deposited as a complex into the yolk of the egg. The importance of this pathway is well illustrated by a strain of Single Comb White Leghorn chickens (*Gallus gallus domesticus*) which lack normal oocytic riboflavin uptake. The allele describing this condition is the *rd* allele. Fertilized eggs from hens homozygous for the *rd* allele are unable to develop into viable embryos. These eggs lack the riboflavin required for normal embryonic development, and as a result the embryo expires on or around the 13th day of incubation. Early investigations found higher than expected levels of riboflavin excreted in the urine of sexually mature homozygous *rd* females (67, 68). This observation led to the hypothesis that the *rd* defect was related to renal riboflavin reabsorbtion, and to the subsequent adoption of the term avian riboflavinuria to describe the condition. However, a later study of isolated kidney sections failed to demonstrate a difference in the renal riboflavin transport of affected (*rd/rd*) vs. normal (*Rd/Rd*) females (69).

When eggs laid by *rd/rd* hens are assayed by fluorescence titration and cellulose acetate electrophoresis, they appear to contain no functional ribBP (44). Therefor, the defect in avian riboflavinuria appears to be a lack of functional ribBP which normally is required for the transport of riboflavin into the egg compartments. The riboflavin bound by ribBP and sequestered in the eggs of normal hens is found in the urine of affected hens. Eggs laid by homozygous mutant hens can be rescued by injecting a minimal amount of riboflavin through the shell (43). This results in normal embryonic development and hatching, demonstrating the biochemical nature of the *rd* defect, and illustrating two important principles: firstly, the absolute requirement for functional ribBP in the reproduction of the chicken ; and secondly, the lack of a requirement for ribBP in the hatched chick which can utilize riboflavin from the diet in the absence of the carrier protein.

Several properties of wild type ribBP may be important for its uptake into oocytes. Its glycosylation, phosphorylation, primary sequence or charge distribution may all play a role in putative receptor-ligand interactions. We were initially intrigued by the possibility that there might exist a mutant form of the protein which was deficient in one of these properties but was not detected by previous analysis. If the mutation resulted in the production of a dysfunctional riboflavin binding protein, characterization of this mutation would provide new insights into the structure-function relationships in the molecule. With this in mind I undertook to clone the mutant gene in an attempt to delineate the molecular basis for this form of avian riboflavinuria, i.e., the defect associated with the *rd* allele.

I. C. 3. RIBOFLAVIN BINDING PROTEINS FROM OTHER SPECIES

Riboflavin binding proteins have been found in many groups of chordates. These include organisms as diverse as fish, reptiles, amphibians, birds, and mammals. In particular riboflavin binding protein are found in carp (*Cyprinus carpio*) (70), alligator (*Alligator mississippiensis*) (71), Indian python (*Python molurus*) (72), painted turtle (*Chrysemys picta*) (72), Japanese quail (*Coturnix japonica*) (73), muscovy duck (*Cairina moschata*) (72), marmosets (*Callithrix jacchus*) (74), bonnet monkey (*Macaca radiata*) (75), rats (76), mice (77) and humans (78).

The riboflavin binding proteins in reptiles and amphibians have amino acid compositions and molecular weights similar to their avian counterparts (72). As well, they all appear to be phosphoglycoproteins. It is likely that they are all coded for by related genes. One could assume that the riboflavin binding proteins in oviparous species could have a conserved function as well, namely to facilitate the transport of riboflavin into the growing oocyte. This appears to be the case since all of these proteins are found, associated with riboflavin, in the eggs of these animals. The function of the mammalian riboflavin binding proteins is less clear. The known mammalian ribBPs are all immunologically related to avian ribBP. The mammalian ribBPs are pregnancy specific, they are only found either in the serum of pregnant females (74), the serum of the developing fetus, or in the amniotic fluid(78). Immunization of mammals with avian ribBP results in the generation of antibodies which are thought to recognize mammalian ribBP. This treatment results in an animal which is unable to carry a fetus to full term. The anti-ribBP antibodies interfere with the delivery of riboflavin to the fetus and as a result the development of the fetus is impaired(79), it is resorbed and pregnancy is terminated. Elucidation of the exact role of mammalian ribBP will undoubtedly tell us a great deal about mammalian reproductive biology.

I. D. RECEPTOR MEDIATED ENDOCYTOSIS

Receptor mediated endocytosis is a mechanism for the cellular uptake of macromolecules in eukaryotic cells. This process is common to more than 25 systems of receptor-ligand combinations, however by far the best characterized is the uptake of LDL by the LDL receptor in human fibroblasts(80). The first step in this process is the binding of the blood-born LDL particle by one of 15,000 LDL receptors located on the surface of the cell(19). The receptors are not distributed randomly on the cell surface but are gathered at specific regions of the plasma membrane(81, 82). These regions are coated with the protein clathrin. Clathrin is a fascinating molecule because of its ability to interact with itself to form a highly organized structure.

Multiple copies of clathrin arrange themselves on the cytoplasmic surface of the plasma membrane such that they have an appearance resembling a woven basket which is visible under the electron microscope. These regions are some 100 to 500 nm in diameter and are restricted to less than 2% of the cells surface. These regions are often referred to as clathrin coated pits. Localization of the LDL receptor in the clathrin coated pit is mediated by a short internalization sequence near the carboxyterminal end of the LDL receptor. The sequence Asn-Pro-Val-Tyr (NPVY) is localized on the cytoplasmic side of the plasma membrane and may interact specifically with the clathrin matrix to hold the receptor in this location(83). Similar sequence elements have been found in other receptors localized in clathrin coated pits and taken up by receptor mediated endocytosis.

The binding of LDL to the LDL receptor is followed by the second step in the receptor mediated endocytosis pathway. The coated pit becomes deeper until the invagination is pinched off from the plasma membrane. The resulting coated vesicle contains LDL bound to the LDL receptor as well as many other receptor ligand complexes. The coated vesicles lose most of their external clathrin coat and as they do so fuse with other endocytotic vesicles forming endosomes. The formation of the endosome is accompanied by an acidification of the interior of the compartment which is driven by an ATP dependent proton pump(84). The pH of the mature endosome is in the range 5.0 to 6.0. As a result of this acidification there is a dissociation of the receptor ligand complex. The free receptor is recycled to the cell surface where it relocates in newly formed coated pits and begins the cycle again. The LDL however is transferred to lysosomes where it is degraded.

The sorting of the LDL receptor away from the lysosomal destined LDL is not well characterized. However the sorting of the asialoglycoprotein receptor has been studied and some useful observations have been made(85). Specifically a subpopulation of endosomes has been identified which features long thin extensions where the receptors are localized. The receptors remain attached to the membrane. It is thought that the receptors are removed from the endosome via budding of the long extensions while the free ligand remains in the lumen. Clearly clathrin may play a role in this process as clathrin has also been shown to be localized in the thin extensions. The name given to the whole structure is the Compartment of Uncoupling of Receptor and Ligand (CURL).

Although many of the features of the LDL receptor system are common to other systems there are some variations to this general theme. In the case of the receptor for epidermal growth factor (EGF), the receptor is not localized to the coated pit until complexed with the ligand(86). As well, there is more than one possible fate for a receptor once in the endosome. In some cases the receptor and the ligand are both degraded. In some cases, for example in epithelial cells, the receptor-ligand complex is transported specifically across the cell to another surface. Not all ligands are destined for degradation in lysosomes. In the case of transferrin, the receptor ligand complex dissociates in the endosome but the ligand transferrin is recycled to the cell surface with the receptor(87). Important for our purposes is the situation in the avian oocyte where the ligand VLDL is partially degraded in the lysosomal particle yet awaits further processing by the embryo.

I. D. 1. THE MAMMALIAN LIPOPROTEIN RECEPTOR FAMILY

I. D. 1. a. THE HUMAN LDL RECEPTOR

The best characterized of the lipoprotein receptors is the human LDL receptor (Fig. I. 4.). The Human LDL receptor was first purified in the laboratory of Drs. Goldstein and Brown by Dr. W. J. Schneider. Cloning of the receptor lead to detailed structural analysis and elucidation of a multi-domain structure which has proven useful in the discussion of any of the members of the LDL receptor family(88). The human LDL receptor consists of five domains after the removal of a signal sequence. These domains, as arranged from the amino terminus, are the ligand binding domain, the EGF precursor homology domain, the O-linked sugar domain, the trans-membrane domain and the intracellular domain (Fig. I. 4.). A brief discussion of each of these domains follows.

Ligand Binding Domain

The ligand binding domain is the domain responsible for interaction with the ligands apo B and apo E. This region consists of 292 amino acid residues at the amino terminus of the receptor. Within this region are seven tandemly organized repeats of a 40 amino acid sequence known as a type A repeat. Within each repeat is the conserved sequence Ser-Asp-Glu (SDE). These negatively charged SDE repeats are thought to interact cooperatively with positively charged regions on apo B (89). While repeats 2 thru 7 are required for apo B binding, the binding of apo E is thought to be mediated mainly by repeat 5. The highly organized structure of the ligand binding domain is maintained by conserved cysteine residues. Within each repeat there are six cysteines which participate in intra-repeat disulfide bonds resulting in the tightly folded structure of each repeat.

Not surprisingly, the highly organized structure of this domain is related to the structure of the gene coding for this region (88). The gene structure is as follows; exon 1 encodes the signal sequence for the receptor, exon 2 codes for the first repeat , exon 3 for repeat 2, exon 4 codes for repeats 3,4, and 5, exon 5 codes for repeat 6 and exon 6 codes for repeat 7. The evolution of the ligand binding domain may have involved the duplication of one initial exon which resulted in the seven repeats found in the modern LDL receptor. Interestingly, the intervening sequences, introns, interupt the reading frame at the same position of the codon. As such the loss or addition of an exon does not disrupt the reading frame and results in the production of a receptor with altered binding characteristics.

EGF Precursor Homology Domain

Adjacent to the ligand binding domain is the 400 amino acid EGF precursor homology domain. This region is named after a striking similarity between parts of this region and the precursor for epidermal growth factor(90). Three regions in particular, designated growth factor repeat A, B and C, contain repeats of cysteine rich sequences called type B repeats. They differ from the type A repeats in that they lack the negatively charged SDE clusters thought to mediate ligand binding. Five of the exons in the LDL receptor are related to exons in the EGF precursor gene. These five exons appear to have resulted from a partial duplication of the gene for the EGF precursor. The A, B and C repeats in the LDL receptor are coded for separately by three of these five exons.

The EGF precursor domain of the LDL receptor may have more than one function. Firstly, repeats A and B may contain Ca^{2+} binding sites which may confer the Ca^{2+} requirement for binding of LDL to the receptor(91). Second, repeat A appears to be involved in LDL binding since deletion of this repeat via site directed mutagenesis results in a reduction of LDL binding(92). Thirdly, the entire domain appears to have a role in the endosomal dissociation of the receptor ligand complex and the subsequent recycling of the receptor to the cell surface(93).

O-Linked Sugar Domain

This domain consists of a stretch of 58 amino acids immediately outside the plasma membrane. This region contains some 18 serine or threonine residues. These hydroxylated amino acids become glycosylated in the course of protein maturation. Initially, N-acetylglucosamine is the sole sugar present but upon processing in the Golgi galactosyl and sialyl acid residues are added. The purpose of this domain and its contribution to the overall function of the LDL receptor is not understood. The entire domain can be deleted without any apparent loss in function (94).

Trans-Membrane Domain

The 22-25 residues which cross the plasma membrane comprise the trans-membrane domain. Typical of trans-membrane domains these residues are hydrophobic and appear to act to anchor the receptor in the membrane. Deletion of this domain results in the secretion of mutant receptor from the cell. This domain is the least conserved of the domains in the LDL receptor family. This would be evidence against any secondary function for the region.

Intracellular Domain

This domain consists of the 50 carboxyterminal residues of the LDL receptor. This region has been shown to be crucial for the localization of the receptor in clathrin coated pits. This in turn is critical for internalization of the receptor ligand complex. By means of naturally occurring mutations and site directed mutagenesis studies a sequence has been identified which seems to be responsible for this activity. The sequence Asn-Pro-Val-Tyr (NPVY) is required for proper internalization of the receptor (83). This sequence has been called the internalization sequence and is present in the form NPXY, where X denotes any amino acid, in all members of the LDL receptor family.

I. D. 1. b. THE HUMAN LDL RECEPTOR RELATED PROTEIN

The second most prominent member of the LDL receptor family is the LDL-receptor-related protein (LRP) (Fig. I. 4.). LRP is synthesized as a 4525 amino acid, 600 kDa polypeptide which undergoes proteolytic processing in the Golgi. The result of this processing is an 85 kDa membrane spanning fragment that remains non-covalently associated with the remaining 515 kDa extracellular subunit (95). LRP contains many of the structural elements of the LDL receptor (96). LRP is presumed to be the primordial lipoprotein receptor. The gene for LRP can be found in organisms as primitive as the roundworm *Caenorhabditis elegans*. It is believed that the LDL receptor evolved as a result of a duplication of the gene for LRP, with this duplicate gene loosing several elements until it evolved into the modern LDL receptor gene (97). The LRP has 31 cysteine rich type A binding repeats which are related to the 7 found in the LDL receptor(96). In the LRP these repeats are arranged in four clusters (Fig. I. 4.). The LRP contains 22 of the EGF precursor-like type B repeats compared to the three found in the LDL receptor. The cytoplasmic domain of the LRP contains two copies of the internalization signal, NPTY and NPVY, responsible for the localization of the LRP in clathrin coated pits on the plasma membrane. The LRP differs from the LDL receptor in that it lacks the O-linked sugar domain found in the LDL receptor. In its place are six copies of a sequence which is related to the EGF precursor-like type B repeats. However these sequences differ from the type B repeats in the arrangement of cysteine residues and more closely resemble sequences in EGF itself. For this reason they are called EGF-like repeats.

Originally the role of LRP was thought to be as a receptor involved in the clearance of chylomicron remnants. It binds apo E containing lipoproteins by virtue of four copies of a type A repeat related to the one in the LDL receptor which binds apo E(98, 99). However the discovery that LRP bound the plasma protein α_2-macroglobulin (α_2-M) brought into question the role of this receptor(100). The LRP is now thought of as a multifunctional receptor which binds a variety of ligands(97, 101). These ligands can be grouped into three main groups. The first group is a group of proteases and their inhibitors which are bound by LRP. These include α_2-M and the various proteases bound by it. As well, LRP has been shown to bind plasminogen activator inhibitor (PAI)-1 when it is complexed with the proteases tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). Free t-PA is bound by LRP as well. The second group of ligands bound by the LRP could be classed as lipoproteins or apolipoprotein-like molecules involved in lipid metabolism. They include the apo E containing chylomicron remnants, VTG and the enzyme lipoprotein lipase. Lipoprotein lipase is thought to bind to LRP by virtue of an amphipathic helix which mimics the similar structures present in apolipoproteins. The third group of ligands would be classed as extra-physiological ligands. These include the bacterial toxin *Pseudomonas* exotoxin A which enters the cell via interaction with LRP (102). Also included in this group are rhinoviruses of the minor group which bind LRP. In addition to these ligands which are bound by LRP, there is a broad spectrum inhibitor of LRP ligand binding known as receptor-associated protein or RAP. RAP is a 39 kDa protein which inhibits the binding of all the known ligands to LRP. The mechanism of this inhibition is not clearly understood but it is thought that the LRP molecule binds multiple copies of RAP and RAP in turn blocks the binding of other ligands to LRP. The physiological function and control of RAP is most likely very complicated if it regulates the binding of the various ligands to this multifunctional receptor.

I. D. 1. c. THE HUMAN VERY LOW DENSITY LIPOPROTEIN RECEPTOR

A recent arrival in the growing family of lipoprotein receptors is the receptor for very low density lipoproteins (VLDL)(103, 104). The genes for the human and rabbit VLDL receptors have been cloned. They are very similar to the genes for the LDL receptor. The arrangement of introns and exons is almost completely conserved between the LDL receptor and VLDL receptor genes. The VLDL receptor has all of the structural elements found in the LDL receptor with the addition of one extra type A ligand binding repeat giving a total of 8 in the VLDL receptor (Fig. I. 5.). The extra binding repeat is coded for by one extra exon in the gene for the VLDL receptor. The gene for the LDL receptor is located on chromosome 19 while that of the VLDL receptor is on chromosome 9. It is very likely that the two genes arose from a gene duplication event. Although the genes are similar their patterns of expression are very different. The LDL receptor is expressed in liver, fibroblasts and the adrenal gland while the VLDL receptor is expressed in heart, muscle and adipose tissue. The VLDL receptor seems to function as a receptor for apo E containing lipoproteins such as VLDL and possibly chylomicrons. This receptor may be responsible for supplying the heart with a rich source of triglycerides in the form of VLDL. It may also mediate the accumulation of fat by adipose tissue and the uptake of VLDL in monocyte/macrophage cells (104).

The VLDL receptor is expressed as one of two variants which are the result of differential splicing. One variant lacks the O-linked sugar domain found in the normal VLDLR and LDLR. The exact function of this domain is not well understood, in fact it can be deleted from the LDL receptor without any apparent loss of function(94). However the presence of these two variants may have some physiological relevance which remains to be elucidated.

I. D. 1. d. GLYCOPROTEIN 330

Glycoprotein 330 (gp330) is a membrane protein which shares many of the structural features of the LDL receptor, LRP and the VLDL receptor(105, 106). It was originally thought to have a molecular weight of 330 kDa but has been recently shown to be as large as 550 kDa (107). It contains both type A and type B cysteine rich repeats. The intracellular domain contains three copies of the internalization sequence NPXY. gp330 is found in clathrin coated pits in epithelial cells from the kidney, yolk sac and epididymis but not in the liver. gp330 is the antigen in the rat autoimmune disease Heymann nephritis (105). This is a disease characterized by an autoimmune attack on the glomerular epithelium resulting in impaired kidney function. Effected animals have circulating autoantibodies against gp330. The normal physiological function of this protein is not known.

I. D. 2. THE AVIAN LIPOPROTEIN RECEPTOR FAMILY

The laying hen is tasked with the synthesis and targeting of large amounts of lipoproteins destined for the growing oocyte and the maintenance of cholesterol homeostasis in the body. It accomplishes this by the use of at least four members of the LDL receptor gene family. The laying hen has a somatic cell LDL receptor, the analog of the human LDL receptor, and a somatic cell LRP similar to the human LRP. The laying hen also expresses two oocyte specific lipoprotein receptors. These include a 380 kDa oocyte specific LRP analog and the 95 kDa receptor for VLDL and VTG.

I. D. 2. a. THE OOCYTE 95 kDa RECEPTOR

The 95 kDa receptor for VLDL and VTG is the best characterized member of the avian LDL receptor family. As early as 1979, receptor mediated endocytosis of VLDL into growing oocytes had been observed by electron microscopy (108). Early attempts to characterize the receptors for VLDL and VTG lead to the erroneous conclusions that apo-II was responsible for VLDL binding (109) and that phosvitin mediated VTG binding and internalization (110). Using techniques which had proven useful in studies of the human LDL receptor, Dr. Schneider and colleagues showed that both VLDL(1) and VTG(2) were taken up by specific receptors and that both ligands bound to the same 95 kDa receptor(6). Binding of VLDL to the 95 kDa receptor is mediated by apo B and binding of VTG to the receptor is mediated by lipovitellin(40). Surprisingly, this receptor has also been shown to bind the mammalian ligand apo E (41). Recent work in our laboratory has shown that this receptor also binds α_2-macroglobulin.

The 95 kDa receptor has been cloned in our laboratory and has been identified as the avian analog to the mammalian VLDL receptor (111). It shares all of the structural elements of the human VLDL receptor (Fig. I. 5.). The tissue distribution of the 95 kDa receptor in chickens is somewhat different from that seen in mammals. Although mRNA for the receptor can be detected via PCR in both heart and striated muscle, immunoblotting experiments show the level of expression in these tissues is very low relative to that of the oocytes. In mammals this receptor is thought to direct the uptake of VLDL into tissues which require triglycerides as a source of energy.

The primary role of this receptor in avian species appears to be directing the oocytic uptake of VLDL and VTG.

I. D. 2. b. THE AVIAN OOCYTE LRP ANALOG

In the process of characterizing the 95 kDa receptor it became apparent that there was another protein present in oocyte membrane extracts which bound VTG. Ligand blotting experiments identified a 380 kDa oocyte membrane protein which was later shown to be related to the human LRP. This protein is referred to as the oocyte LRP(112).

The oocyte LRP has been purified and partially characterized on a biochemical level. Using a solid phase filtration assay Stifani et al. measured high affinity saturable binding of VTG to this protein (K_d ~ 40 nM). This receptor also binds human lipoproteins, in particular it has been shown to bind apo B 100 containing human LDL and human apo E. Although immunologically distinct from the other members of the avian lipoprotein receptor family, the oocyte LRP shares some of the structural features of other lipoprotein receptors. Amino acid sequence analysis of three tryptic peptides revealed high homology to the human LRP. Further structural analysis will become possible once the cDNA coding for this protein has been cloned. This work is presently ongoing.

I. D. 2. c. THE AVIAN SOMATIC CELL LRP

The avian somatic cell LRP is better characterized on the structural level than the oocytic LRP. The full length (15,598 bp) cDNA for the avian somatic LRP has been cloned(113). It codes for a 4,522 amino acid protein which is very closely related to the human LRP with 85% identity in the amino acid sequence. The avian LRP contains 31 type A repeats arranged in clusters which mirror those of the human LRP. Curiously, the region which is most closely conserved between the two species is the EGF precursor domain.

This domain in the LDL receptor is thought to be involved in the endosomal dissociation of the receptor ligand complex and receptor recycling. The role of this domain in the LRP is not well understood but its conservation through evolution may imply an important function. The two internalization sequences (NPXY) found in the cytoplasmic domain of the human LRP are identical in the avian protein. The avian LRP is also proteolytically cleaved after synthesis. In the human LRP the site of this cleavage is the amino acid sequence RHRR. The sequence RNRR is found at the expected cleavage site in the avian protein.

One of the proposed functions of the mammalian LRP is the uptake of chylomicron remnants via interaction with apo E. However, as mentioned previously, chickens do not have chylomicrons, nor do they synthesize apo E. The primary role of the LRP in avian species may involve the uptake of α_2M. The avian somatic LRP has been shown to bind avian α_2M. Both avian somatic LRP and the human LRP have been shown to bind VTG in ligand blotting experiments. The implications of VTG, a yolk precursor, binding to the somatic LRP, are not well understood.

I. D. 2. d. THE 130 kDa LDL RECEPTOR ANALOG

Cell culture studies of chicken embryo fibroblasts (CEF) identified a somatic cell LDL receptor with a molecular weight of 130 kDa (114). This receptor binds and internalizes apo B containing lipoproteins and appears to regulate cholesterol homeostasis in somatic cells in the same way that the human LDL receptor does. Preliminary cDNA cloning has identified a short clone which aligns with the EGF precursor domain of the human LDL receptor(115). At the nucleic acid level this clone shows a 68% homology to the human LDL receptor gene and when translated shows a 66% homology at the amino acid level. This receptor seems to be the avian analog of the human

LDL receptor. It remains to be seen how the expression and activity of this receptor is regulated. Clearly the expression of all the members of the avian LDL receptor family must be strictly controlled so as to best facilitate the reproductive effort of the laying hen. This control may differ significantly from that in the mammalian system.

I. E. RNA SPLICING

In order to provide the reader with information necessary to understand the implications of some of the results presented in this thesis, the following pages are intended to give a brief summary of RNA splicing in eukaryotes. Most eukaryotic transcripts contain both coding and non-coding sequences. The splicing process removes the non-coding intronic sequences and joins the coding exons forming a mature transcript which is then transported to the cytoplasm for translation. This process is directed by sequence elements within the immature message (Fig. I. 6.). These sequence elements are strongly conserved in organisms as diverse as yeast and mammals (116). The 5′ or donor splice site lies at the 5′ end of an intron within a 9 nucleotide (nt) consensus sequence. The 3′ or acceptor splice site lies at the 3′ end of an intron within a 15 nt consensus sequence. The 3′ consensus contains a pyrimidine rich stretch 5′ to the 3′ splice acceptor sequence. A third site, the branch point, is a conserved A which lies within 18-38 nt upstream of the 3′ splice site within the less strongly conserved branch point consensus. The study of these sequences has lead to a greater understanding of the splicing mechanism.

I. E. 1. THE SPLICEOSOME

Splicing occurs within a large complex, the spliceosome. The spliceosome contains five small nuclear ribonucleoprotein particles (snRNPs); U1, U2, U4, U5 and U6. Each snRNP contains a RNA component as well as several polypeptides. The snRNPs and several other polypeptide splicing factors (117) associate with the immature transcript to form the spliceosome. The initial step in spliceosome assembly is thought to be the binding of U1 snRNP to the 5′ splice donor site (Fig. I. 6.). This binding is facilitated by the base pairing of the 5′ end of the U1 snRNA to the highly conserved 5′ consensus sequence. U1 snRNP has been shown to bind to the 5′ donor site in the absence of ATP or other snRNPs (118). U1 binding is thought to be followed by binding of U2 snRNA to the branch point region. This region is usually 10 - 50 nt upstream from the 3′ splice acceptor site. The branch point region is recognized in part by U2 snRNP in an ATP dependent binding reaction. U2 binding is preceded by and requires binding of at least one additional factor, U2AF, consisting of 2 polypeptides which bind to the polypyrimadine tract downstream from the branch point site (119). U2AF acts together with at least two other non-snRNP polypeptide factors, SF1 and SF3, mediating U2 snRNP binding (120). Wu and Manley have reported that U2 also interacts directly with the branch site via base pairing at the pre-mRNA (121). The interaction of the pre-mRNA with U1, U2 and the various other factors presumably induces bending in the pre-mRNA bringing the 5′ splice donor site into close proximity to the branch site and the 3′ splice acceptor site.

The next step in spliceosome assembly involves the addition of a tripartite 25S U4/U5/U6 complex (Fig. I. 6.) (122). U4 and U6 snRNPs interact with one another primarily via base pairing within their snRNAs. U5 is thought to interact with the U4/U6 snRNP via protein-protein interaction. The U4/U5/U6 complex interacts with the pre-spliceosome via pre-mRNA bound U1 and U2 snRNPs (123) as well as directly via the pre-mRNA. U5 snRNP has been shown to interact with the pre-mRNA directly at both the 5′

and 3′ splice sites [Newman, 1992 #499] and to have a role in 5′ splice site selection (124). The complete spliceosome structure is thought to be influenced further by the interactions of several other partially characterized splicing factors (117). The first step of the two-step splicing pathway occurs only after assembly of the mature spliceosome.

The first step of the splicing pathway is the cleavage of the 5′ splice site (Fig. I. 6.). This generates a free exon and a structure known as a lariat. A lariat results from the formation of a 2′ - 5′ phosphodiester bond between the conserved G residue at position 1 of the intron and 2′ hydroxyl of the conserved A residue at the branch point. The second step of the splicing pathway is the cleavage of the 3′ splice acceptor site, ligation of the two exons and release of the excised intron which retains its lariat conformation (Fig. I. 6.) (117).

I. E. 2. SEQUENCE MEDIATED DISRUPTIONS OF THE SPLICING PATHWAY

The importance of the 5′ splice donor sequence is demonstrated in cases where the sequence is not conserved. In these cases splicing occurs either inaccurately or not at all (125, 126, 127, 128, 129). The sequence in this region is known to interact directly with the 5′ end of U1 snRNA via sequence complementarity. Inaccurate splicing is often the result of cryptic splice site activation. A cryptic splice site is a site which resembles the normal 5′ splice site and becomes activated when the normal site is mutated becoming less complementary to U1 snRNA. The location of cryptic splice sites can often be predicted from their complementarity to U1 snRNA (130).The effects of most mutations in the 5′ splice site can be suppressed by compensatory base changes in U1 snRNA (131). HeLa cells transfected with plasmids containing adenovirus genes which have point mutations in the 5′ splice sites express defective splicing products. However when these same cells are cotransfected with mutant U1 genes which contain compensating base changes in the 5′ end of U1 snRNA they express wild type splicing products. This treatment is not sufficient to alleviate the effects of mutations at position 1 of the intron. Siliciano and Guthrie examined the efficiency of various mutant 5′ splice sites in the presence of compensating U1 snRNA and found that 5′ donor G to A mutations could not be rescued by compensating mutations in U1 snRNA(132). This would seem to indicate a role for the 5′ G which extends beyond U1 recognition and binding.

I. F. SCOPE OF THE THESIS

This study was begun in 1988 at which time very little was known about the uptake of ribBP into the growing oocyte. Experiments by Drs. Barber and Stifani had demonstrated the existence of specific receptors for VLDL and VTG, but at that time it was not known that these two yolk precursors were taken up by the same receptor. It therefor seemed likely that there may be yet another specific receptor for ribBP. At the onset of this study the cDNA for ribBP had yet to be cloned. Intrigued by the possibility that the disease riboflavinuria may be caused by a defect in the ligand ribBP which effected its putative receptor mediated uptake, we chose to determine the nature of the *rd* allele. The experiments presented in this thesis were designed in an attempt to reach the following goals:

1. Elucidate the mechanism by which ribBP is taken up into the growing oocyte, in particular demonstrate the existence or lack of a specific receptor for ribBP in the oocyte plasma membrane.
2. Delineate the molecular basis of the inherited disease avian riboflavinuria, i.e., the defect associated with the *rd* allele.

3. Evaluate the results of these investigations in light of what is already known about riboflavin binding proteins and/or oocyte growth in avian and other species.

Figure I. 1.

Morphology of the laying hen follicle. The primary oocyte lies within a complex multilayered structure named the follicle. Yolk precursors enter the follicle via the heavily vascularized theca cell layer surrounding the oocyte. Inside this layer is the the basal lamina, which separates the theca cells from the granulosa cells. Separating the granulosa cells from the oocyte is the perivitelline layer. Yolk precursors entering the follicle from the circulation must move through the theca cell layer, cross the basal lamina and pass between the granulosa cells before coming into the perivitelline layer and eventually contacting the oocyte plasma membrane. Clustered on the membrane surface in clatharin coated pits are specific receptors for VLDL and VTG and possibly other yolk precursors.

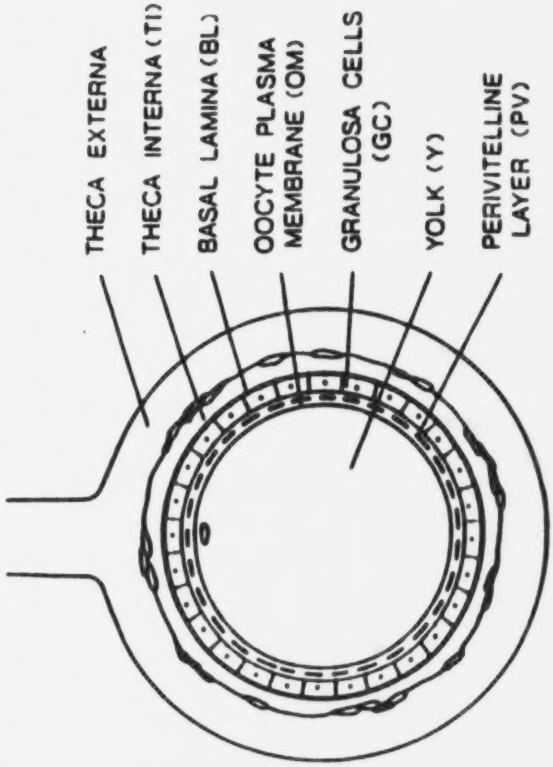

THECA EXTERNA
THECA INTERNA (TI)
BASAL LAMINA (BL)
OOCYTE PLASMA MEMBRANE (OM)
GRANULOSA CELLS (GC)
YOLK (Y)
PERIVITELLINE LAYER (PV)

Figure I. 2.

Oogenesis in the chicken. The yolk precursors VLDL, VTG and ribBP are synthesised in the liver in response to estrogen. After entering the circulation, precursors are taken up in the ovary by growing oocytes. The mature oocyte is released from the follicle and enters the oviduct at the infundibulum. As the oocyte moves through the oviduct the albumin is deposited. Following shell deposition the egg is layed.

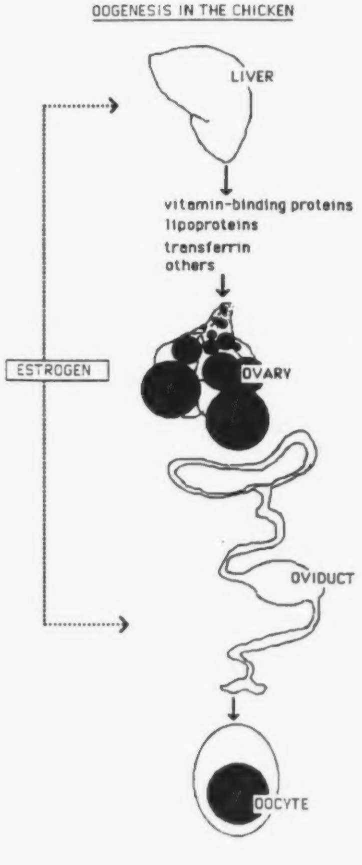

OOGENESIS IN THE CHICKEN
LIVER
vitamin-binding proteins
lipoproteins
transferrin
others
ESTROGEN
OVARY
OVIDUCT
OOCYTE

Figure I. 3.

Structural features of chicken riboflavin binding protein. The cDNA for riboflavin binding protein codes for a 221 amino acid protein after the removal of a 17 residue signal peptide. Two carboxy-terminal arginines predicted by the cDNA sequence are not found in the mature protein. Riboflavin binding protein is post-translationally modified by glycosylation at asparagines 36 and 147, and by phosphorylation at 8 serine residues clustered near the carboxy-terminus. Upon deposition in the oocyte, specific proteolytic cleavage at leucine 206 or lysine 208 results in the removal of the carboxy-terminal 11 or 13 residues.

Riboflavin Binding Protein

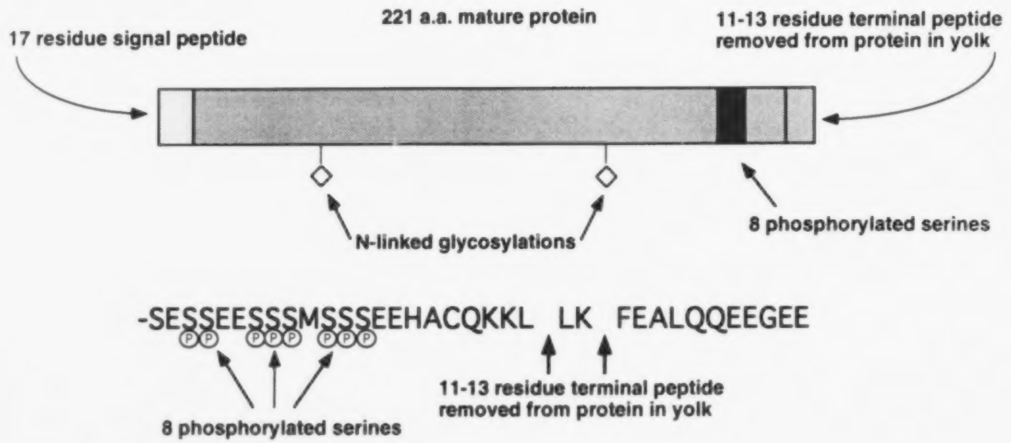

17 residue signal peptide
221 a.a. mature protein
11-13 residue terminal peptide removed from protein in yolk
N-linked glycosylations
8 phosphorylated serines
-SESSEESSSMSSSEEHACQKKL LK FEALQQEEGEE
P P P P P P P P
8 phosphorylated serines
11-13 residue terminal peptide removed from protein in yolk

Figure I. 4.

Structural schematic of the human LDL receptor and LRP. The human LDL receptor consists of five domains. These domains, as arranged from the amino terminus, are the ligand binding domain (filled ellipses representing cysteine rich type A binding repeats), the EGF precursor homology domain (line bound by open circles representing EGF precursor-like type B repeats), the O-linked sugar domain (open box), the membrane spanning region (filled box) and the intracellular domain (lined). Also shown is the location of the NPVY internalization motif. LRP has 31 cysteine rich binding type A repeats (filled ellipses) arranged in four clusters. LRP contains 22 of the EGF precursor-like type B repeats (open circles). LRP lacks the O-linked sugar domain found in the LDL receptor. In its place are six copies of a sequence which is related to the EGF precursor-like type B repeats (filled circles). Also shown are the two copies of the internalization motif, NPTY and NPVY.

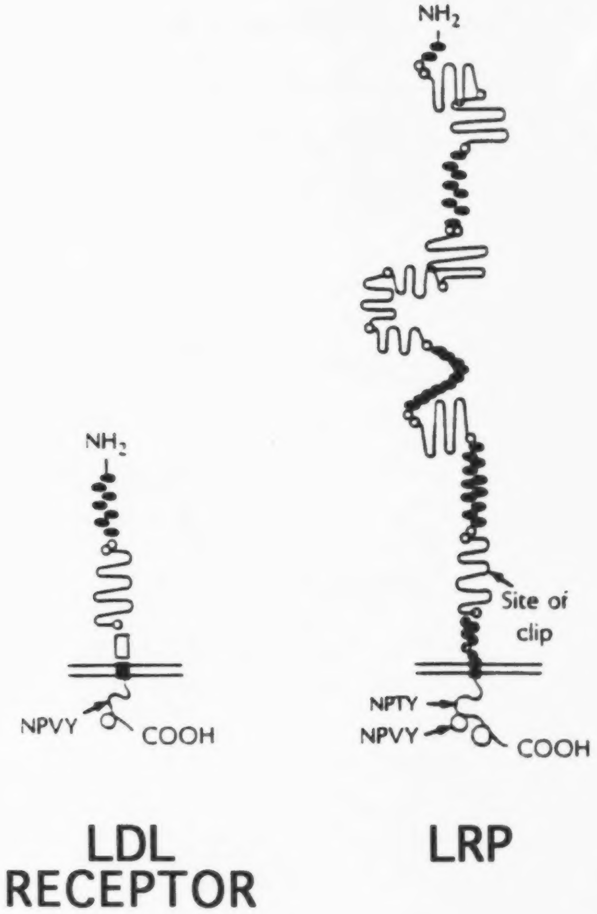

NH$_2$
NPVY
COOH
LDL RECEPTOR
NH$_2$
Site of clip
NPTY
NPVY
COOH
LRP

Figure I. 5.

Structural schematic and comparison of the chicken 95 kDa oocyte membrane receptor (VLDLR), the human VLDL receptor and human LDL receptor. Numbers refer to percentage identity between the chicken 95 kDa receptor amino acid sequence and the human VLDL and LDL receptors. Both the chicken 95 kDa receptor and the human VLDL receptor have all of the structural elements found in the LDL receptor with the addition of one extra type A ligand binding repeat. While the chicken 95 kDa receptor is expressed on the oocyte plasma membrane, the human VLDL receptor is expressed in heart, muscle and adipose tissue, and the human LDL receptor is expressed in liver, fibroblasts and the adrenal gland. The human VLDL receptor is expressed as one of two variants which are the result of differential splicing. One variant lacks the O-linked sugar domain found in the normal VLDLR and LDLR. The chicken 95 kDa receptor as expressed on the surface of the oocyte plasma membrane lacks the O-linked sugar domain.

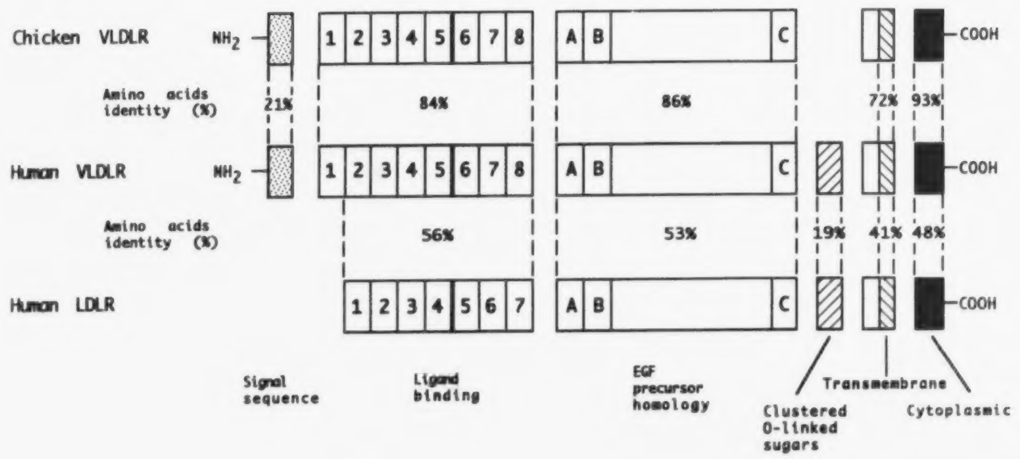
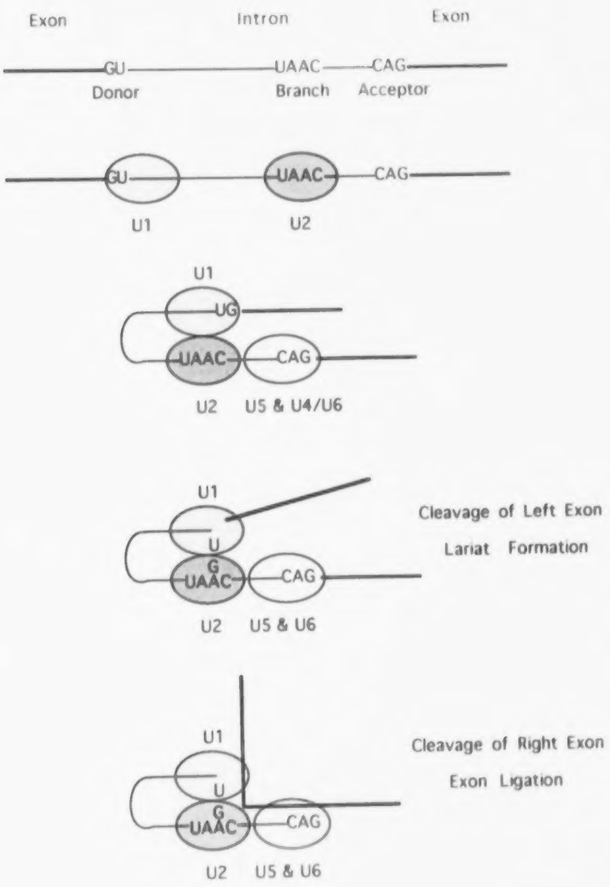

Chicken VLDLR
NH2
1 2 3 4 5 6 7 8
A B
C
COOH
Amino acids identity (%)
21%
84%
86%
72%
93%
Human VLDLR
NH2
1 2 3 4 5 6 7 8
A B
C
COOH
Amino acids identity (%)
56%
53%
19%
41%
48%
Human LDLR
1 2 3 4 5 6 7
A B
C
COOH
Signal sequence
Ligand binding
EGF precursor homology
Transmembrane
Clustered O-linked sugars
Cytoplasmic

Figure I. 6.

The eukariotic splicing pathway. The splicing process removes the non-coding intronic sequences and joins the coding exons forming a mature transcript. This process is directed by conserved sequence elements within the immature message. The 5′ or donor splice site lies at the 5′ end of an intron within a 9 nucleotide (nt) consensus sequence. The 3′ or acceptor splice site lies at the 3′ end of an intron within a 15 nt consensus sequence. The 3′ consensus contains a pyrimidine rich stretch 5′ to the 3′ splice acceptor sequence. A third site, the branch point, is a conserved A which lies within 18-38 nt upstream of the 3′ splice site within the less strongly conserved branch point consensus. The initial step in spliceosome assembly is thought to be the binding of U1 snRNP to the 5′ splice donor site, facilitated by the base pairing of the 5′ end of the U1 snRNA to the 5′ consensus sequence. U1 binding is thought to be followed by binding of U2 snRNA to the branch point region. The next step in spliceosome assembly involves the addition of a tri-partite 25S U4/U5/U6 complex. Following spliceosome assembly, the first step of the two-step splicing pathway is the cleavage of the 5′ splice site, generating a free exon and a lariat structure. The second step of the splicing pathway is the cleavage of the 3′ splice acceptor site, ligation of the two exons and release of the excised intron which retains its lariat conformation.


Exon
Intron
Exon
GU
UAAC
CAG
Donor
Branch
Acceptor
GU
UAAC
CAG
U1
U2
U1
UG
UAAC
CAG
U2
U5 & U4/U6
U1
U
G
UAAC
CAG
U2
U5 & U6
Cleavage of Left Exon
Lariat Formation
U1
U
G
UAAC
CAG
U2
U5 & U6
Cleavage of Right Exon
Exon Ligation

CHAPTER II

MATERIALS AND METHODS

II. MATERIALS AND METHODS

II. A. MATERIALS

Octyl-ß-D-glucoside, Triton X-100, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, 17α-ethinylestradiol, Freund's complete adjuvant, Freund's incomplete adjuvant, peroxidase conjugated protein A, and bovine serum albumin were obtained from Sigma. Protein and nucleic acid molecular weight standards were from BRL. Nitrocellulose paper BA 85 was from Schleicher and Schuell, NH or from Amersham. Nylon membranes were from Amersham. Restriction enzymes were from Pharmacia or from BRL. Sodium (^{125}I) iodide (11-17 mCi/mg) was from Edmonton Radiopharmaceutical Centre, Edmonton, Alberta or from Dupont. γ^{32}P labeled dCTP was from Dupont. Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) was from Pierce Chemical Co. or from Sigma. Suramin (sodium salt) was from FBH Pharmaceuticals, NY. CNBr-activated Sepharose 4B (No. 17-04300-01), protein A-Sepharose CL4B, DEAE Sepharose, Mono Q, Sepharose 6 and Sephadex G-25 PD 10 columns were from Pharmacia. DEAE-cellulose DE 52 was obtained from Whatman. A recombinant λgt11 clone containing riboflavin binding protein cDNA, *rd* tissue samples and *rd* serum samples were all obtained from Dr. H. B. White III, University of Delaware.

II. B. ANIMALS AND DIETS

Wildtype White Leghorn layers (8-18 months old) and roosters were obtained from local poultry farms. Hens were maintained on layer mash, roosters on grower mash, both with a light period of 12 hours. Homozygous *rd* layers were from a White Leghorn flock maintained at the University of Delaware, Newark, DE, USA. Oocytes were collected during slaughter by permission of Lilydale Poultry Sales, Edmonton, Alberta. Antibodies were raised in adult female New Zealand White rabbits.

II. C. PURIFICATION OF RIBOFLAVIN BINDING PROTEINS

Riboflavin binding protein was purified from the serum of estrogen treated roosters, laying hen serum, egg yolk and egg white by a modification of a previously published protocol (133). Roosters were estrogenised by intramuscular injection of 10 mg/kg body weight of 17 α-ethinylestradiol dissolved in propyleneglycol. Birds were sacrificed two days later by decapitation and blood was collected into 0.6 volumes of 0.5 M sodium citrate, pH 5.5. A cocktail of protease and phosphatase inhibitors was added to the samples so that they contained; 100 µg/ml PMSF, 2 nM leupeptin, 2.5 µg/ml aprotinin, 1.0 ng/ml pepstatin A, 20 mM NaF and 40 mM 4-Nitrophenol-PO_4. Egg yolk and egg white samples were diluted with 0.6 volumes 0.5 M sodium citrate, pH 5.5 and centrifuged for ten min. at 5000 x g. The supernatant was collected and all samples were then treated as follows. The samples were adjusted to 45% saturation by the addition of solid ammonium sulfate and stirred for half an hour at 4 °C. This was followed by centrifugation for 10 min. at 5000 x g. after which the supernatant was collected and adjusted to 85% saturation by the addition of solid ammonium sulfate. This was stirred for 30 min. at 4°C and centrifuged for 30 min. at 16,000 x g. The resulting floating pellet was dissolved in 50 mM sodium acetate pH 6.0, and dialyzed overnight against distilled water and centrifuged for 10 min. at 3000 x g to remove any precipitate. The samples were adjusted to pH 6.0 with 1.0 M acetic acid and loaded onto DEAE sepharose G-50 pre-equilibrated with 0.05 M sodium acetate, 0.1 M NaCl, pH 5.5. The column was washed with several column volumes of the same buffer and the bound protein was eluted with 0.05 M sodium acetate, 0.5 M NaCl, pH 5.5. This eluate was diluted with 4 volumes of 0.1 M Na Acetate pH 5.8 and loaded onto an FPLC Mono-Q column. The bound ribBP was eluted with a gradient ranging from this buffer to 0.1 M Na Acetate, 0.5 M NaCl, pH 3.7.

II. D. PURIFICATION OF VITELLOGENIN

Vitellogenin was purified from laying hen plasma. All operations were carried out at 4 °C. Ten ml of blood was drawn from the wing vein and collected on ice into tubes containing reagents giving the following final concentrations: 16 mM sodium citrate, 1 mM PMSF, 2 nM leupeptin, and 2.5 μg/ml aprotinin. Blood was centrifuged at 3,000 x g for 15 min. The resulting supernatant was centrifuged at 200,000 x g for 2 hr. The high speed infranatant fluid that separated from the low density fraction was subjected to anion-exchange chromatography on DEAE-cellulose. The DEAE-cellulose matrix was packed into 30 x 1.9 cm columns and washed with 200 ml of 100 mM sodium citrate, 2 mM $CaCl_2$, 1 mM PMSF, 0.2 % Triton X-100, pH 5.5. The column was equilibrated with 200 ml of 50 mM sodium citrate, 2 mM $CaCl_2$, 1 mM PMSF, pH 5.5. Ten to fifteen ml of the subnatant fluid was applied to the column, which was then washed with 200 ml of 100 mM sodium citrate, 2 mM CaCl2, 1 mM PMSF, pH 5.5. VTG was eluted in a linear salt gradient (100 ml) from 0 to 300 mM NaCl in 100 mM sodium citrate, 2 mM CaCl2, 1 mM PMSF, pH 5.5. VTG-containing fractions were pooled and dialyzed at 4 °C against 150 mM NaCl, 2 mM $CaCl_2$, and 5 mM Tris-HCl pH 7.8. After dialysis, leupeptin (2 nM), aprotinin (2.5 μg/ml), and PMSF (1 mM) were added to reach the indicated concentrations and the protein was stored at -70 °C .

II. E. PREPARATION OF MEMBRANE EXTRACTS

Oocyte membranes were prepared as described previously (1). Briefly, ovarian follicles were collected during slaughter and transferred to an ice cold buffer of 20 mM Tris-HCl, 1 mM $CaCl_2$, 150 mM NaCl, 1 mM PMSF and 2 nM leupeptin pH 8.0 (buffer A). Connective tissues and theca cell layers were removed, yolk contents were extruded and the remaining membranes and granulosa cell layers were washed in ice-cold buffer A. The membranes were minced, homogenized and centrifuged at 5,000 x g for 5 minutes to remove large debris. The resulting supernatant was centrifuged at 100,000 x g for one hour and the resulting membrane pellets were resuspended in buffer A and centrifuged again at 100,000 x g for one hour. These membrane pellets were either stored at -70°C or extracted with either 1% Triton X-100 or 36 mM octyl-glucoside.

Membrane pellets (10-20 g) were resuspended in 4.5 ml 250 mM Tris-maleate, 2 mM $CaCl_2$, 1 mM PMSF, 5 nM leupeptin, 2.5 µg/ml aprotinin pH 6.0. Volume was brought up to 9 ml by the addition of water and either Triton X-100 to yield a 1% Triton X-100 solution or octyl-glucoside to yield a 36 mM octyl-glucoside solution.. The membranes were incubated at 4°C for ten min. before centrifugation at 100,000 x g for one hour. The supernatant, Triton X-100 oocyte membrane extract or octyl-glucoside extract, was then stored at -70°C.

II. F. ANTIBODY PREPARATION

II. F. 1. ANTI-ribBP ANTIBODIES

FPLC pure serum ribBP was subjected to SDS PAGE (134), staining with 0.05% Coomasie Blue and destaining with H_2O. The ribBP band was cut out from the gel and crushed in 1 ml of buffered 50 mM Tris-HCl, 250 mM NaCl, 2 mM $CaCl_2$ and 0.1% SDS pH 7.5. The resulting slurry was suspended in 4 ml of this buffer and incubated at 23 °C for 16 hrs. The buffer was collected and the eluted protein precipitated by the addition of 4 volumes of ice-cold acetone followed by incubation at -70 degrees for one hour. The sample was then spun for 15 min. at 3000 x g and the resulting pellet was resuspended in 500 µl of phosphate buffered saline. The resulting protein concentration was determined by the Lowry method (135).

Polyclonal antibodies against ribBP were raised by injection of 150 mg of the above purified serum ribBP in phosphate buffered saline which was emulsified with an equivalent volume of Freund's complete adjuvant (136) on day 0, followed by injections at days 14, 28 and 35 with 150 mg of gel purified serum ribBP in Freund's incomplete adjuvant. The rabbit was bled on day 48 and once every week thereafter. The antibodies against vitellogenin, the 95 kDa oocyte membrane receptor and oocyte LRP were as previously described(6).

II. F. 2. IgG ISOLATION USING PROTEIN A SEPHAROSE

Serum from 50 ml of rabbit blood was repeatedly passed over a protein A-Sepharose CL-4B column which had been pre-equilibrated in 0.1 M NaH_2PO_4, pH 8.0. The column was washed with 3 column volumes of the same buffer. Bound IgG was eluted with 1 ml fractions of 1.0 M Acetic acid, 0.1 M Glycine into tubes containing 0.4 ml of 1M NaH_2PO_4, pH 7.4. The eluted fractions were pooled and adjusted to 40% saturation with saturated ammonium sulfate solution and incubated at 23°C for 30 min.. The IgG was sedimented by centrifugation at 15000 x g for 15 min, dissolved in 10 ml phosphate-buffered saline and then dialyzed vs. phosphate-buffered saline overnight at 4°C.

II. F. 3. AFFINITY PURIFICATION OF ANTI-ribBP IgG

Egg white ribBP (10 mg, Sigma) was coupled to CNBr-activated Sepharose 4B as per the manufacturers instructions. A column of ribBP-Sepharose was prepared, washed with ten column volumes of 10 mM Tris, pH 7.5, and 5 mg of anti-ribBP IgG were loaded on the column. The column was washed with 20 column volumes 10 mM Tris pH 7.5, followed by 20 column volumes of 10 mM Tris, 0.5 M NaCl pH 7.5. The bound ribBP specific IgG was eluted with fractions of 100 mM glycine, pH 2.5 into tubes containing one tenth of the fraction volume 1.0 M Tris, pH 8.0. The column was then washed with 10 mM Tris, pH 8.8, followed by 100 mM triethylamine, pH 11.5, followed by 10 mM Tris, pH 7.5. The fractions containing protein were pooled and concentrated as above via ammonium sulfate precipitation. The IgG was resuspended in phosphate buffered saline and stored at -20°C.

II. G. ELECTROPHORESIS AND TRANSFER TO NITROCELLULOSE

One dimensional SDS electrophoresis was carried out according to Laemmli on 4.5% to 18% gradient slab gels unless otherwise indicated in the figure legends (134). The oocyte membrane extracts used in ligand blotting experiments did not contain reducing agents nor were they heated prior to loading. When indicated in Fig. legends other samples were reduced by the addition of 10 mM dithiothreitol and were heated to 90°C for five min. prior to application. Gels were calibrated with Bio-Rad broad range molecular weight standards (161-0317) containing: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg white ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; and aprotinin, 6.5 kDa. Gels were stained in 0.1% (w/v) Coomassie Blue, 5% (v/v) acetic acid, 25% (v/v) methanol for one hour then destained with 10% acetic acid.

Electrophoretic transfer of proteins to nitrocellulose was performed in 25 mM Tris and 192 mM glycine. Transferred proteins were stained with 0.2% Ponceau S in 3% TCA and destained with distilled water.

II. H. LIGAND BLOTTING

Ligand blotting experiments were performed in a buffer composed of either 20 mM Tris-HCl , 90 mM NaCl, 2 mM $CaCl_2$ pH 7.4, or 25 mM HEPES, 100 mM NaCl, +/- 1mM $CaCl_2$, +/- 1 mM Na_2HPO_4 pH 7.4. The incubations with labeled ligands were performed after blocking the membranes for one hour in a solution of 5% (w/v) in the above buffer. Labeled ligands were incubated in blocking buffer for 2 hours at room temperature and the free ligand was removed by washing in buffer which was free of BSA. The concentrations of ligands used in each experiment are indicated in the figure legends as are the times of autoradiography. Autoradiography was at -70 °C.

II. I. IMMUNOBLOTTING

Nitrocellulose was blocked for one hour in 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween-20 and 5.0% non- fat dry milk pH 7.5 as per the protocol supplied with the Amersham ECL western blotting kit (RPN 2106). Antibodies were added to the incubations as indicated in the Fig. legends and visualized with 50 ng/ml Protein A-peroxidase conjugate (Sigma P 8651) followed by ECL reagents as per the manufacturers protocol. In some experiments the blocking step was omitted and the first incubation was in 50% serum, 50% 20 mM Tris pH 7.4, 90 mM NaCl and 2 mM $CaCl_2$. In some experiments the nitrocellulose was stripped of antibody and Protein A-peroxidase by submerging in 100 mM β -mercaptoethanol, 2% sodium dodecyl sulfate and 62.5 mM Tris-HCl for 30 min. before reprobing with an antibody against VTG. Autoradiographs were obtained by exposing the nitrocellulose to Dupont Reflection™ NEF- film for the times indicated in the Fig. legends.

II. J. CHEMICAL CROSSLINKING

Serum from estrogen treated roosters was diluted 1:20 with PBS. 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide Hydrochloride in PBS was added to a final concentration of 12 mM and the sample was incubated for 16 hr at 20° C. The sample was then subjected to SDS page, transferred to nitrocellulose and probed with immunopurified antibody against ribBP.

II. K. SERUM CHROMATOGRAPHY

A Pharmacia Superose 6 HR 10/30 column was equilibrated with 20 mM TRIS, 90 mM NaCl, 2 mM $CaCl_2$ with or without 20 mM EDTA pH 7.4 at a flow rate of 0.4 ml min^{-1}. Aliquots of serum from estrogen treated roosters were filtered through Millipore Millex-HA filters (pore size of 0.45 µm) and then centrifuged in an Eppendorf microfuge at 13,000 rpm for 1 min. Aliquots of 25 µl were loaded onto the column and 1 ml fractions were collected. The fractions were precipitated by the addition of 30 µl of 1.0 % sodium deoxycholate followed by 0.5 ml of 50% trichloroacetic acid. The samples were incubated for 15 min. on ice and then centrifuged for 15 min. at 3000 rpm. The supernatant was decanted and the protein pellets were washed with 0.5 ml of ice-cold acetone. The dried pellets were resuspended in 150 µl of O'Farrell's buffer and run on SDS page (137). The gels were transferred to nitrocellulose membranes which were probed with the immunopurified antibody against ribBP to visualize ribBP in the eluted fractions.

II. L. RADIOLABELLING

Radioiodination was performed using iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril) as an oxidant(138). 0.5 mCi of ^{125}I was added to 8.25 µg of serum ribBP in 250 mM Na_2HPO_4 pH 7.5. The labeled protein was separated from free iodine by gel filtration chromatography. The specific activity of the ^{125}I labeled riboflavin binding protein is indicated in the Fig. legends. Autoradiographs of ligand blots were obtained by exposing nitrocellulose to Fuji RX film for the times indicated in the Fig. legends.

II. M. ASSAY FOR ribBP BINDING TO OOCYTE MEMBRANE EXTRACTS

An assay was developed to quantitate the binding of serum ribBP to oocyte membrane extracts. Oocyte membrane octyl-glucoside extracts were precipitated on ice by lowering the concentration of octyl-glucoside below the critical micellar concentration by the addition of 7 volumes of 50 mM Tris-maleate, 2 mM $CaCl_2$ pH 6.0. The precipitate was collected after centrifugation at 100,000 x g for 1 hr at 4°C. The pellet was resuspended on ice by aspiration through a 22 guage needle in 2.8 ml of ice-cold 20 mM Tris-HCl, 50 mM NaCl, 2 mM $CaCl_2$, 16 mg/ml bovine serum albumin pH 8.0 (incubation buffer). Aliquots of 70 µl of this preparation were set aside and either 20 µl of H_2O or 20 µl of 20 mM EDTA pH 8.0 were added to each. Serial dilutions of ^{125}I-labeled sribBP were prepared and added to each incubation in 10 µl aliquots. Incubation was for 90 min. at 23°C. After this time 60 µl aliquots of each incubation were layered onto 130 µl of fetal bovine serum. Free ligand was separated from receptor-ligand complex by centrifugation in a Beckman Airfuge at >100,000 rpm for 20 min. The pelleted receptor-ligand complex was washed with 175 µl of fetal bovine serum followed by centrifugation for 5 min. at >100,000 rpm. The tips were cut from the centrifuge tubes and the precipitated ^{125}I-labelled sribBP was quantitated.

II. N. PROPAGATION OF PLASMIDS

The λgt11 ribBP cDNA clone was digested with *Eco*RI and subcloned into the plasmid pTZ18. The resulting plasmid, pTZ18IM1, was propagated in *E. coli* JM109. The PCR primers used for amplification of cDNA contain *Eco*RI sites to facilitate the cloning of their products. The PCR-amplified cDNAs were digested with *Eco*RI and purified from low melting point agarose after electrophoresis. The fragments were ligated into pTZ19 previously cut with *Eco*RI and dephosphorylated. These constructs were used to transform electro-competent JM109 by electroporation. The PCR-amplified genomic DNA was ligated directly into a pCR™ 1000 vector using the protocol supplied by the manufacturer (Invitrogen Corporation). This construct was used to transform *E. coli* INVαF′. Transformants were selected, and plasmid DNA from these cells was digested with *Eco*RI and ligated into *Eco*RI-cut pTZ19 to facilitate the construction of nested deletions using the exonuclease III method (139).

II. O. DNA AND RNA ISOLATION

DNA was isolated from liver by the method of Davis et. al. (140). Total RNA was isolated by the guanidinium isothiocyanate method (141) from oviduct or liver tissue which had been quick-frozen in liquid nitrogen. Poly(A)$^+$-RNA was purified from total RNA by oligo(dT) cellulose chromatography (142, 143).

II. P. SOUTHERN AND NORTHERN ANALYSIS

Genomic Southern blotting was performed on 8μg of wild type or mutant DNA (140) digested with the respective restriction enzyme. The DNA was resolved by 1.0% agarose gel electrophoresis and transferred to Hybond N™ (Amersham RPN.303N). Northern analysis was performed on total RNA which was resolved on a 1.2% agarose gel containing 3.0% formaldehyde. After electrophoresis the RNA was transferred to Hybond N™. Hybridizations were performed in 50% (wt/vol.) formamide, 6x SSC, 1x Denhart´s solution, 0.1 mg/ml salmon sperm DNA, 100 mg/ml dextran sulfate, 0.1% $NaDodSO_4$ at the temperatures indicated in the Figure legends.

II. Q. cDNA PREPARATION AND PCR

cDNA was prepared using poly(A)$^{+}$-RNA. cDNA synthesis was primed with an excess of oligo(dT)$_{15}$ (Boehringer Mannheim 813 702) and the reaction was driven with Superscript™ Moloney Murine Leukemia Virus reverse transcriptase (Bethesda Research Laboratories, 8053SA) as per the manufacturer's instructions in the buffer supplied. cDNA produced in such a manner was used as template for PCR reactions. PCR was carried out on both genomic and cDNA using primers directed against the wild-type ribBP cDNA (144). The primers used for amplification of the cDNA, ribBP Pos 1 and ribBP Neg 1, have the sequences;

5´ CTC GAA TTC CAG AAG GAC AGC AAA AGA GGA 3´ and
5´ GGG AAT TCG ATT TAT TGT GTT CTC AGA AGT GAG 3´,
respectively.

The underlined portions of the sequences correspond to the regions complementary to the respective target sequence. These primers contain 5´ *Eco*RI sites to facilitate cloning and manipulation of their products.

II. R. GENOMIC DNA PCR

The primers used for amplification of genomic DNA, ribBP Pos 2 and ribBP Neg 2, are flanking the 100 bp deletion in the *rd* cDNA and have the sequences;

5´ GGG AAT TCA TGA ATG CAC TCT GTA TTC T 3´ and
5´ GGG AAT TCG TGC GGA GAA CAC CGG TAA 3´.

The underlined portions of the sequences correspond to the regions complementary to the respective target sequence. These primers contain 5′ *Eco*RI sites to facilitate cloning and manipulation of their products.

II. S. SEQUENCING AND DATA ANALYSIS

Sequencing of the cDNA clones was accomplished in both directions via the dideoxy-chain termination method using Sequenase™ (USB). The full sequences were determined by the construction and sequencing of a series of nested deletions using the exonuclease III method (139). Sequencing of the genomic clones was accomplished in both directions using an Applied Biosystems 373A automated DNA sequencer in combination with the exonuclease III method. Ambiguities in the genomic sequences were resolved by manual sequencing using oligonucleotide primers and the Sequenase™ method. Contiguous sequences were arranged and analyzed using the program Geneworks™ running on an Apple Macintosh computer.

II. T. OTHER METHODS

Protein concentrations were determined by the Lowry method (135). Protein concentrations of lipid or detergent containing samples were determined by a modification of the Lowry method involving precipitation of the samples in the presence of sodium deoxycholate and trichloroacetic acid.

CHAPTER III.

CHARACTERIZATION OF THE MOLECULAR DEFECT ASSOCIATED WITH THE *rd* ALLELE

III. CHARACTERIZATION OF THE MOLECULAR DEFECT ASSOCIATED WITH THE *rd* ALLELE

III. A. INTRODUCTION

The examination of naturally occurring disruptions in biological processes often yields useful information which can in turn be used to build models to describe the normal processes. The most obvious example in receptor biology is the genetic disease familial hypercholesterolemia (FH). Many of the details of the LDL receptor pathway were discovered during studies of this disorder (88). Studies of the mutant restricted ovulator (*ro*) strain have taught us a great deal about normal oocyte growth (145). Likewise, the study of the *rd* mutant strain helped build the model for ribBP function that exists today (43, 44, 69). When the cDNA for avian ribBP was cloned in 1988 it became possible to consider a molecular investigation into the nature of avian riboflavinuria (46). The information available to us at that time indicated that the most likely explanation for the phenotype was either a defect in, or a loss of, the gene for ribBP. The phenotype could be the result of either the expression of a disfunctional ribBP, which had yet to be detected, or a total loss of ribBP expression in the homozygous mutant hen.

In order to determine which of these two scenarios were responsible for the *rd* phenotype I used two approaches to the problem. The first approach involved a rigorous examination and comparison of the genes for ribBP in both mutant and wild type hens. The second approach involved the development of a highly specific, yet polyclonal antibody which recognized ribBP. Such an antibody would be expected to recognize any mutant ribBP produced assuming the conservation of at least one epitope. This antibody was used in immunoblotting experiments comparing the ribBP content of mutant and wild type serum, egg yolk and egg white. The results of these experiments not only agreed with previous investigations but confirmed the results of the molecular studies. Using these techniques I have shown that avian riboflavinuria is caused by a mutation in the non-coding region of the gene for riboflavin binding protein and that the result of this mutation is that there is no ribBP in the serum, egg yolk or egg white of hens homozygous for the *rd* allele.

III. B. RESULTS

III. B. 1. THE *rd* MUTATION

To determine the level of ribBP message in the *rd* mutant, I performed a Northern blot analysis on RNA from homozygous mutant and normal animals (Fig. III. 1). Using radiolabeled full-length ribBP cDNA as a probe, very little, if any message for ribBP was detected in the *rd* hen. As described in the Materials and Methods section, PCR was used to amplify the protein coding region of this message from cDNA derived from normal and mutant liver mRNA. The reactions resulted in amplification of a 875 bp product in the *Rd* and a 775 bp product in the *rd* , respectively. These PCR products were subcloned into pTZ19 and sequenced. The results are shown in Fig. III. 2. Sequence comparison shows that the PCR products were derived from the riboflavin binding protein cDNA, with some deviations from the published sequence (46). Both the *Rd* and *rd* cDNAs cloned here have a T at nucleotide 93 and therefor code for an asparagine residue at position 14 of the mature protein. The published cDNA sequence for ribBP (46) , with a G at nucleotide 93, codes for a lysine at this position, whereas peptide sequencing of ribBP (45) showed a single amino acid polymorphism with either lysine or asparagine at position 14. I concluded that I had cloned the cDNA for the asparagine isoform of ribBP. The *rd* cDNA contains an additional polymorphism in the 3′ non-coding region at nucleotide 753 where a C replaces the wild type T.

Expectedly, the most notable deviation from the published sequence is in the *rd* message which contains a 100 nt deletion relative to the wild type. The *rd* deletion is in the coding region of the cDNA, removing nucleotides 140 - 239 from the *rd* message. The 100-bp deletion was not detected by Southern analysis of *RdRd* and *rdrd* genomic DNA using a full length *Rd* ribBP cDNA as a probe (see Fig. III. 3). Therefor, it seemed likely that the loss of this region from the *rd* transcript could be due to a splicing defect, possibly induced by a sequence element within the intronic DNA. In order to investigate this possibility, I generated genomic clones containing this portion of the ribBP gene. PCR using primers flanking the deletion resulted in the amplification of a 2.1 kb genomic fragment in both the *Rd* and the *rd* alleles (see Fig. III. 4). This fragment contains portions of three separate exons. Since little is known about the structure of the gene for ribBP I arbitrarily designated these exons. The 5′ end of the genomic clone contains 26 bp of the 3′ end of one exon which, for the purposes of this study, is designated exon 1. This is followed by an intron of 820 bp, IVS1, followed by an exon of 100 bp, designated exon 2. Exon 2, present in both the normal and the mutant genomes, corresponds exactly to the 100 bp deletion found in the *rd* cDNA clone. Exon 2 is followed by 1095 bp of intronic DNA, IVS2. The 3′ end of the genomic clone contains 53 bp of a third exon, exon 3.

It has been demonstrated that mutations in sequences near splice junctions can affect splicing efficiency (126, 146). Thus, the splice junctions and branch point sequences of the *Rd* and the *rd* alleles were compared to the consensus sequences for such regions (see Fig. III. 6). With respect to these regions, the sequences from the two genotypes were identical with one exception, a G to A mutation at position 1 of the 5′ splice donor site of IVS2, shown in Fig. III. 4, III. 5 and III. 6. The 5′ GT is essentially invariant among functional splice sites (116). Among unconventional 5′ splice donor sites which were collected in an analysis of 3724 5′ functional splice junctions in release 57.0 of GenBank there were only 31 splice sites which did not have the 5′ GT and only 11 that did not retain the G(116) . This statistical evidence alone indicates an essential role for the conserved G in the splicing mechanism. The role of the 5′ G is thought to be an interaction with U1 small nuclear ribonucleoprotein particle (snRNP). U1 snRNP binds to the 5′ splice site via base pairing with the free 5′ end of U1 snRNA (131). In those 5′ splice sites which match the consensus sequence for splice donors, U1 snRNA will base pair with 8 contiguous nucleotides (cf. Fig. III. 6). Splice sites which deviate from this consensus are thought to compete less well for U1 snRNA (147).

The other splice sites in the region cloned are not particularly unusual. The two 3′ splice acceptor sites essentially match the consensus for splice acceptor sites in that they contain a pyrimidine-rich sequence followed by the sequence CAG. Branch point sequences are normally 10 to 50 nucleotides upstream of the 3′ splice acceptor sequence (116). The site of branch formation lies within a moderately well conserved consensus sequence of five nucleotides, CTAAC. The fourth nucleotide of the branch point consensus is the A involved in lariat formation. A single-stranded region, strongly conserved from yeast to mammals (148), between two stem-loop structures in U2 snRNA base-pairs with the pre-mRNA at this consensus sequence (121). At position -45 relative to the splice site, IVS2 contains a site which closely matches the consensus for branch points. The potential for this region to base-pair with U2 snRNA is unusually high. The complementary region in IVS2 extends 3 nucleotides beyond the region of pre-mRNA normally thought to base-pair with the U2 snRNA (see Fig. III. 6). A potential branch point in IVS1 at position -42 would allow for base pairing of two nucleotides to U2 snRNA. The sequence CTAACAG lies immediately upstream of the 3′ splice site in IVS1 and has the potential for base pairing with five nucleotides in U2 snRNA; however, it would be highly unusual for a functional branch point to lie within the 3′ splice donor site.

The other intronic polymorphisms in the region cloned are not expected to affect the expression of the *Rd* gene product (see Fig. III. 4).

III. B. 2. IMMUNOLOGICAL RESULTS

Two antibodies were raised against ribBP. The first of these was raised against a commercially available preparation of wribBP. This antibody, although it had a high affinity for ribBP, was not specific enough for our purposes (data not shown). A second antibody was raised against FPLC purified sribBP and was subsequently purified by affinity chromatography on wribBP-sepharose (see Material and Methods). When used in immunoblotting experiments with enhanced chemiluminescent reagents this antibody recognised one band in serum, egg yolk and egg white. This band comigrated with purified ribBP. Fig. III. 7. shows the results of an immunoblotting experiment comparing the ribBP content of the serum, egg yolk and egg white of both wild type and homozygous *rd* hens. This experiment clearly shows the ribBP in the three samples from the wild type animal. This experiment also demonstrates the abscence of any ribBP in the serum, egg yolk and egg white of hens homozygous for the *rd* allele.

III. C. DISCUSSION

Our results indicate that the *rd* phenotype is caused by the G to A mutation in the 5′ splice site of the intron designated IVS2. This mutation abolishes wild type splicing and produces an internally truncated form of *Rd* mRNA. This truncated message is either produced very inefficiently or is unstable, since it is difficult to detect on Northern blots of *rd* mRNA (see Fig. III. 1). These results can be compared to studies of the human β-globin gene (149). Mutations in the human β-globin gene have been shown to be responsible for several types of β-thalassemias (150). The β-globin gene consists of three exons in a structure analogous to the portion of the *Rd* gene under consideration here. In particular, a G to A transition at position 1 of IVS2 is responsible for one type of β-thalassemia (151).

In studies of the transcription and splicing of transiently expressed human β-globin genes in HeLa cells a β-globin gene containing a G to A transition in position 1 of IVS2 was efficiently transcribed and polyadenylated. However, the predominant mRNA, containing a portion of IVS2, was a product of cryptic splice site activation. A minor RNA species which lacked E2, designated E1-E3, was the result of splicing E1 directly to E3. In other studies of the effects of several mutations on the *in vitro* and *in vivo* splicing of the rabbit β-globin gene, RNA transcripts were prepared *in vitro* using the SP6 system (152) in which a G to A mutation at position 1 of IVS2 yielded E1-E3 RNA as the major product(146). A super-lariat which resulted from linking the 5′ splice donor of IVS1 to the branch point of IVS2 could be isolated. However, when this gene was transiently expressed in HeLa cells, no E1-E3 RNA was detected, instead cryptic splice sites were activated which replaced the mutant 5′ donor of IVS2. These results led their authors to propose a "first come, first served" model in which the sequential nature of pre-mRNA synthesis was coupled to spliceosome assembly to favor the formation of "committed" pre-splicing complexes. These complexes presumably predetermine the fidelity of the E1-E2 splice sometime before the splice acceptor site in E3 is made available to the splicing machinery. However, our data show that the major *rd* splicing product *in vivo* is the product of Exon1-Exon3 splicing. E1-E3 product has also been found *in vivo* in the 5′ G to A mutant in human β-thalassemia (151). Therefor it seems that under certain conditions E1-E3 splicing competes with the activation of cryptic splice sites.

Aebi *et al*. (146) constructed a truncated β-globin gene which had E1, IVS1 and part of E2 removed. They showed that when the competing upstream 5′ donor in IVS1 was removed, blocking E1-E3 formation, the G to A mutation did not prevent the first step of splicing. 5′ intron cleavage and lariat formation proceeded, yet the second step, 3′ cleavage and exon ligation, was blocked. The resulting product was a dead-end lariat intermediate containing both the intron and the downstream intron. Presumably the invariant G plays a role in the processes involved in the second step of splicing by facilitating the interactions required for 3′ cleavage or exon ligation.

Recently a role for U5 snRNA in the second step of splicing has been demonstrated (153). It was found that the dead-end lariat intermediates which were the products of G to A mutations in yeast could be processed normally in the presence of mutant U5 snRNA. This processing correlated with mutations which permitted base pairing between a conserved loop in U5 snRNA and nucleotides in the exon adjacent to the 3′ splice donor sequence. Newman and Norman do not propose that this interaction normally directs lariat processing, rather that when the pre-mRNA contains a G to A mutation, the normal positioning of U5 relative to the 5′ end of the exon is perturbed. This positioning can be redirected by base pairing to nucleotides adjoining the 3′ splice site. This would support the idea that the invariant G could involve an essential interaction with U5 snRNP, either through snRNA or one of the other components of the particle.

These related studies help explain the results obtained in the current investigation. The G to A mutation in *rd* would be expected to decrease the affinity of the region for U1 snRNA and lower the efficiency of the first splicing step. However, those pre-mRNAs which proceed through the first step of splicing of IVS2 would be unable to continue through the second step, and the resulting dead-end lariat intermediates would explain the low level of message detected by traditional northern analysis (Fig. III. 1). Exon skipping could be achieved via interaction of the 5′ donor of IVS1 with the 3′ acceptor of IVS2, resulting in the production of the mRNA cloned here.

The results indicate that avian riboflavinuria, as characterized by a lack of riboflavin binding protein, is caused by a mutation in the non-coding region of the gene for riboflavin binding protein, in particular a G to A mutation in position 1 of the intron following the 100 bp exon. The immunological results are in agreement with the molecular data. Fig. III. 7. shows that there is no detectable ribBP found in the serum, egg yolk or egg white of hens homozygous for the *rd* allele.

Figure III. 1.

Northern blot analysis of riboflavin binding protein transcripts in laying hen liver. Total liver RNA was prepared from normal *(Rd)* and homozygous mutant *(rd)* hens as described in Materials and Methods (Chapter II). 0.5 µg of *Rd* RNA and 10 µg of *rd* RNA were loaded on the gel. Hybridization was at 42°C with ^{32}P-labeled *Eco*RI fragment of pTZ18IM1 containing the full length cDNA for ribBP. The positions of migration of the chicken 18 and 28 S ribosomal RNA's are shown on the right. Autoradiography was for one and a half hours.

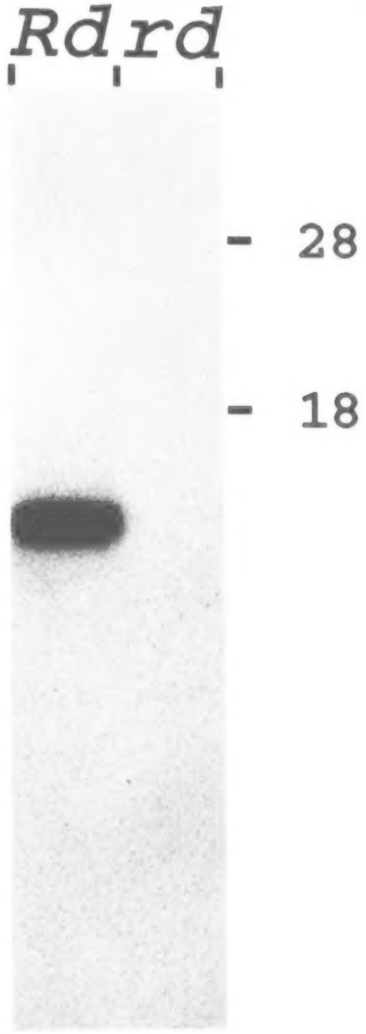

Rd
rd
28
18

Figure III. 2.

Nucleotide and corresponding amino acid sequence of PCR-derived cDNA clones for riboflavin binding protein. The deduced amino acid sequence is shown below the nucleotide sequence. Numbering of nucleotides begins with the initiation codon. Numbering of amino acids refers to the amino terminus of the mature wildtype protein. The 100 nucleotides missing from the *rd* cDNA clone are indicated in lower case (140 - 239). The underlined regions indicate the positions of the PCR primers as described in Materials and Methods (Chapter II). The *arrowhead* indicates the T at position 93, in a triplet coding for asparagine, while the previously published sequence has a G at this position, coding for lysine. The *rd* cDNA has a C at position 753 in the 3′ non-coding region.

```
                                                    1                                                               50
CCC AGA AGG ACA GCA AAA GAG GAA AAG AAG GGA ACA ACA ATG CTG AGG TTT GCC ATC ACC CTC TTT GCT GTC ATC ACA TCA TCT ACC TGC
                                                    Met Leu Arg Phe Ala Ile Thr Leu Phe Ala Val Ile Thr Ser Ser Thr Cys
                                                   -17
                                                    ▽           100
CAG CAG TAT GGA TGT CTG GAA GGG GAC ACC CAC AAA GCG AAT CCA AGT CCT GAG CCA AAC ATG CAT GAA TGC ACT CTG TAT TCT GAA Tct
Gln Gln Tyr Gly Cys Leu Glu Gly Asp Thr His Lys Ala Asn Pro Ser Pro Glu Pro Asn Met His Glu Cys Thr Leu Tyr Ser Glu Ser
1                                   10                                      20                                      30
    150                                                                     200
tcc tgt tgc tat gca aac ttc aca gag caa ttg gct cat tcc cca ata att aaa gta agc aac agc tac tgg aac aga tgt ggg cag ctc
Ser Cys Cys Tyr Ala Asn Phe Thr Glu Gln Leu Ala His Ser Pro Ile Ile Lys Val Ser Asn Ser Tyr Trp Asn Arg Cys Gly Gln Leu
                                    40                                      50                                      60
                        250                                                                     300
agt aaa tcC TGT GAA GAT TTC ACA AAG AAA ATC GAG TGC TTT TAC CGG TGT TCT CCG CAC GCT GCT CGC TGG ATC GAT CCC AGA TAT ACT
Ser Lys Ser Cys Glu Asp Phe Thr Lys Lys Ile Glu Cys Phe Thr Arg Cys Ser Pro His Ala Ala Arg Trp Ile Asp Pro Arg Tyr Thr
                                    70                                      80                                      90
                                    350                                                                     400
GCT GCT ATT CAG TCT GTT CCA CTG TGT CAG AGC TTC TGT GAT GAC TGG TAT GAA GCC TGC AAA GAT GAT TCC ATT TGT GCT CAT AAC TGG
Ala Ala Ile Gln Ser Val Pro Leu Cys Gln Ser Phe Cys Asp Asp Trp Pyr Glu Ala Cys Lys Asp Asp Ser Ile Cys Ala His Asn Trp
                                    100                                     110                                     120
                                                    450                                                             500
CTG ACG GAC TGG GAA CGG GAT GAA AGT GGA GAA AAC CAC TGT AAG AGT AAA TGC GTA CCA TAC AGT GAG ATG TAT GCA AAT GGG ACC GAC
Leu Thr Asp Trp Glu Arg Asp Glu Ser Gly Glu Asn His Cys Lys Ser Lys Cys Val Pro Tyr Ser Glu Met Tyr Ala Asn Gly Thr Asp
                                    130                                     140                                     150
                                                                    550
ATG TGC CAG AGT ATG TGG GGG GAA TCC TTT AAG GTG AGC GAA TCC TCC TGC CTC TGC TTG CAA ATG AAC AAG AAG GAC ATG GTG GCA ATC
Met Cys Gln Ser Met Trp Gly Glu Ser Phe Lys Val Ser Gly Ser Ser Cys Leu Cys Leu Gln Met Asn Lys Lys Asp Met Val Ala Ile
                                    160                                     170                                     180
        600                                                                 650
AAG CAC CTC CTC TCC GAA AGC TCA GAG GAA AGC TCC AGT ATG AGC AGC AGT GAG GAG CAC GCC TGC CAA AAG AAA CTC CTG AAG TTT GAG
Lys His Leu Leu Ser Glu Ser Ser Glu Glu Ser Ser Ser Met Ser Ser Ser Glu Glu His Ala Cys Gln Lys Lys Leu Leu Lys Phe Glu
                                    190                                     200                                     210
                        700                                                                     750
GCA CTG CAG CAA GAG GAA GGG GAA GAG AGA AGA TGA ATT TTG GTG GAT GAA TAT CAG GAG GAG AGG AAT CAT TGT GGA GGT TGT GCT CGG
Ala Leu Gln Gln Glu Glu Gly Glu Glu Arg Arg                                                     C
                                    220
                                        800
GGC ATC ACA GCA GCC TGT CTT ATC CCT CAC TTC TGA GAA CAC AAT AAA TC
```

Figure III. 3.

Southern blot analysis of genomic DNA from normal and mutant hens. 8 μg of wild type (*Rd*) or homozygous mutant (*rd*) DNA was digested with *Pst*I or *Hin*dIII and analyzed by hybridization as described in Materials and Methods (Chapter II). Hybridization was at 42°C with ^{32}P-labeled *Eco*RI fragment of pTZ18IM1 containing the full length cDNA for normal riboflavin binding protein. The migration positions of DNA size markers (kb), are shown on the right. Autoradiography was for 48 hours.

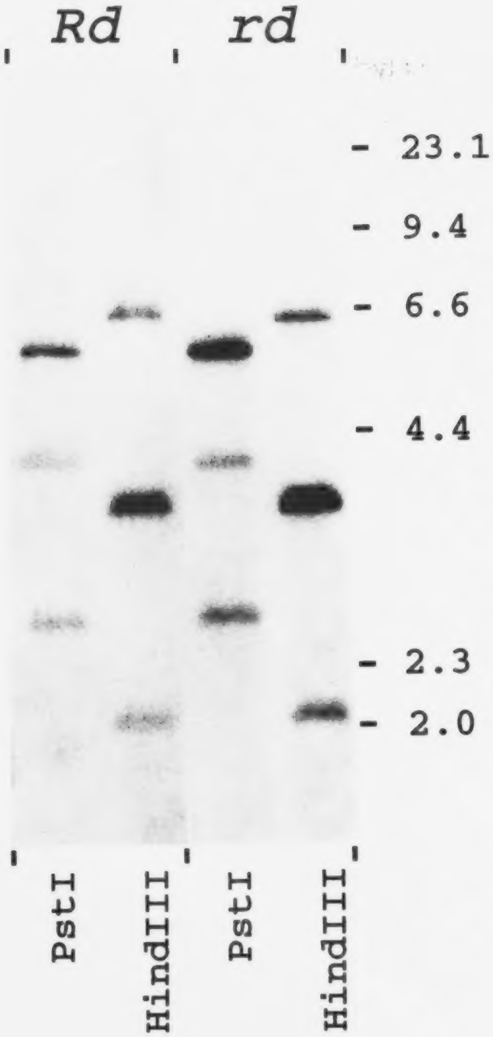

Rd
rd
23.1
9.4
6.6
4.4
2.3
2.0
PstI
HindIII
PstI
HindIII

Figure III. 4.

Nucleotide sequence of PCR derived clones of genomic DNA spanning the 100 bp exon in riboflavin binding protein. The observed wildtype sequence is shown in full with the analogous sequence from the *rd* hen shown below only where it deviates from the wild type. The G to A mutation responsible for the *rd* phenotype is at position 947. Those portions of the sequence which are within exons are *underlined*. *Arrows* point to sequences which are similar to those found at branch points as discussed in the text, and indicated in Fig. III. 6. The polymorphism in the coding region at nucleotide 2066 is conservative for isoleucine. *Dashes* denote deletions.

```
1     GCATGAATGC ACTCTGTATT CTGAATGTAA GAGTGGTTTA GTTTTTGCTG GAGCTTGTTT AGAAGTTCAA TTCTGTATGC ACTGATTGTT CTGGCCATTC  100
                                                    C
101   CTTTAGCTAC TGAAAAATAT ACTAGATGCA GTACAAGTTG CATTGGAAAA ACCTCCCTCA ATGTCATTTT GCTGCATGCA GCTCAAGAGT AAGACTCTGG  200
                                                                                                             --
201   TCCTTAACAA AGTCAGCTGT AAAACGCCCA CTGGCTTTAC TTACTGTTTA AAAAGCTGAT ACAGTTTACT TTTCTGGAAT CTCCATCTGC TTTAAAAAGA  300

301   TTACAGCAAA GGTGGATTCA GCCTATTGTC ATAAAGTGTA CAAATATTTA AAACATGTAT TTTTCTCTTG ATTGCACTGG TGAATTAACT ATGGCCCCCA  400
                                              A
401   TTAAATTATT CAAAATGCAG TTTTTAATGA ACTATAGCAG ATTGCAATTG CATGTAGTTT TCCACACAAA TTGCAAAAGG TTAAACTTTC GATGCTGAGC  500
                                          G                                                                 A
501   AAATTAGAGT AATCATGTTT TTTACATTTC TTCAATTCTC TTTTCCTGTT GGGTAACTTT TAGAATCACT ATCCGTCAGC ACATAACATC TGGCAGAGCA  600
                                                                                     T A          G
601   GTAGTAAACT TCCCAGGGCT TCCTGGACAC ACCTTTACAA AGCAGTGTTC ATGCCAGTCT ATCCCACTTA GCATTTACAC TGCTGCTTCC AAAAACAAAA  700

701   ACATCAGAGC TCGGCTGCAT CTTCTGGTTA GTACCAAGAG ACTTTATCAT GTTACGGACC AAAGAGATTC ACCTTATATT TTTAGCCTTC CAAACAGAAT  800
          ↓                                                    ↓
801   GTTAAAGAAA ATCCTTACTG TAAGATCTTT GTTTCATTCC TAACAGCTTC CTGTTGCTAT GCAAACTTCA CAGAGCAATT GGCTCATTCC CCAATAATTA  900
                                            ↑
901   AAGTAAGCAA CAGCTACTGG AACAGATGTG GGCAGCTCAG TAAATCGTAA GTCGGCTTTG ATGTTGTGTG TGTCCCGGTT CACTAGAGCT ATGCTGTAGC  1000
                                                        A
1001  AGCCACACGG CTAACATTAA CTCCCCACAG ACGGACTGTC CCTACGTTGC CCTAAAGAGA ACGCCGCTTG GTCCGGTTCC CATCATCTCT CCTCTACACC  1100

1101  AGAGCAACAT CTGGCTCTTC TTTTTCCCCT CTCACAAAAG CTCATGAATC CTTAAGTATA TACTTTTTGT GCAGAGAGAT TTATGCCTCT TTCTCAGGAA  1200

1201  AGGTTTATGC TTTTTAAAGC TTGCTTTCAA TATAGAACCA GATCTGGCAA TGCAAAGCAG TCATAAAGCC AGTAGGTAAT GATGACACTG CACCTCTGCT  1300

1301  ACAGAGTAAC CATCACATTC CAGTGAGTGC AAACCCTCGT CAGTAATGAG TTGAGAAAGA GATCAACCAG ATGATCACAT CTTCCAAACA AAATGCACAA  1400
                                                                     C                                         G
1401  GAGGCCAGAA ATAAAACTTC TGAACATTTA GAATAAAAAA TCTTGTAGTC CTGTACATTT GGCAACCATG GAGAATTGGA AAATGTCTTC ATGATTCAGA  1500
                                                                          T              C    G            A
1501  TTTTCAATAC CCTGTCAGAT GCTAGAGCTA TGAAACATGG CAAGTTAGAG AAATAAAAGA ATGAAAGTAG AAAACAATTA AGCAAAAACA GATGTGTGGA  1600

1601  AGTCACAGCG ATTGCCTGCC ACTCCCAGAA GAGCAGCAAC ACTGTTTTTA AGCTATTTCA GCTGGGCTGT GGGAAAATAT AAAAGATGGG TGAAACCATT  1700
                                                                                              -
1701  AGACTGCAAC TTAGAGAATA ATTTGTACTA TAGTTCTAAA CTACATTTTA TCGCGGTCTT GAGCCATTTG TGCCAGGAAA ACTCAAGGCA CCAAAAGATT  1800
                                                                                                                T
1801  ACGGAAAAGC TACACAAATA GGGCTGAAAT ATGCATGGAA ACATTTGTGC ACTCTTTAGT TGGCTATGGT AATCGCACAG AGTCTGTACA CAATACCAAA  1900
                          G
                                                                                                               ↓
1901  ACAATGATAC AAACAGGGCA AAATGATGCT TTTCTCCTAG CCTGTAGTAC TGCATCCACA TCAAAGCAGG TCTAGAAATT GGAAGGTGTA GCCTTAACAC  2000

2001  TGAGAAACGT GTTTATTTGA GGCTTTTCTC ACCCACAACA GCTGTGAAGA TTTCACAAAG AAAATTGAGT GCTTTTACCG GTGTTCTCCG CACG        2094
                                                                             C
```

Figure III. 5.

DNA sequencing gel showing the mutation in the gene for riboflavin binding protein responsible for the *rd* phenotype. Sequences are from PCR derived clones of wild type (*Rd*) and homozygous mutant (*rd*) genomic DNA. The sequence is to be read 5′ to 3′ upwards with respect to the coding strand. The G to A mutation which follows the 100 base pair exon is indicated.

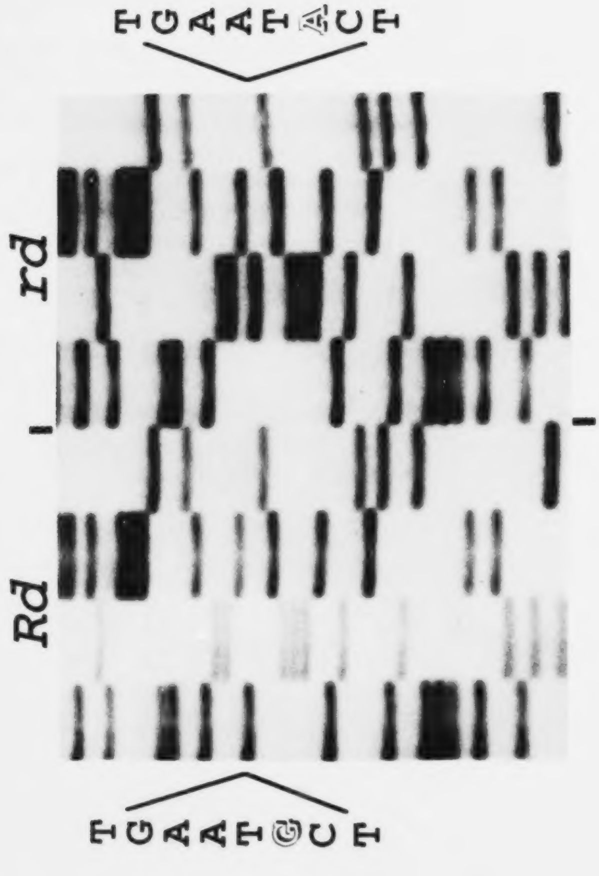

TGAATACT
rd
Rd
TGAATGCT

Figure III. 6.

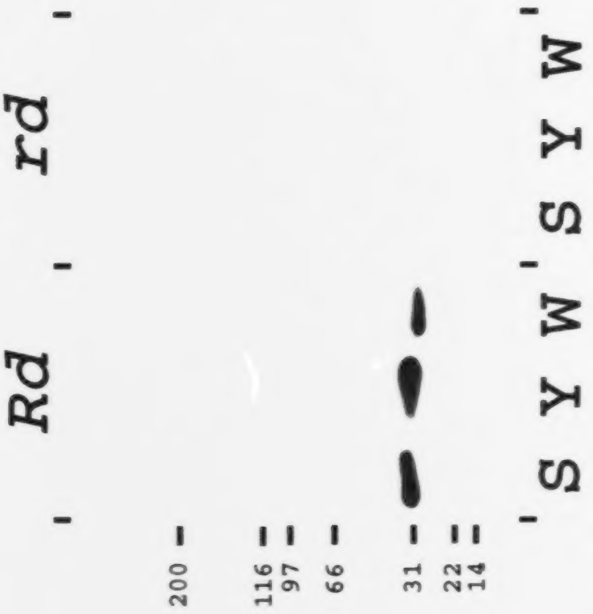
Sequences of interest in the gene for riboflavin binding protein. Intron-exon designations are shown at the top of the Fig. Accepted consensus sequences for splice donor, acceptor and branch point sequences are shown with the analogous sequences in the riboflavin binding protein gene. Regions of the snRNAs U1 and U2 which have the potential to base-pair with the riboflavin binding protein pre-mRNA splice donor and branch point sequences are also shown. The *arrows* point to sequences which are similar to those found at branch points as mentioned in the text and indicated in Fig. III. 4.

Type of Sequence	Exon 1	IVS1	Exon 2	IVS2	Exon 3
Consensus Sequences	......AG	GTAAGT.....CTAAC.....(nPyr)NCAG	G.....AG	GTAAGT......CTAAC.......(nPyr)NCAG	G......
Rd Sequence	......AT	GTAAGA...TGTTAAAG....TTCCTAACAG	C.....TC	GTAAGT....CCTTAACACT....CCCACAACAG	C......
rd Sequence	......AT	GTAAGA...TGTTAAAG....TTCCTAACAG	C.....TC	**A**TAAGT....CCTTAACACT....CCCACAACAG	C......
snRNA Base Pairing	......UC	CAUUCA.....GAU-GUGA.....GAU-GUG	A.....UC	CAUUCA......GAU-GUGA...............	....

↑ ↑ ↑

Figure III. 7.

Western blot analysis of riboflavin binding proteins in serum, egg yolk and egg white of normal (*Rd*) and homozygous mutant (*rd*) laying hens. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and incubated in 2.6 µg/ml affinity purified rabbit IgG raised against serum riboflavin binding protein. Riboflavin binding protein was visualized with protein A linked horseradish peroxidase via the ECL method. Lanes marked S, Y and W contained 200, 40 and 17 µg of total serum, egg yolk and egg white proteins respectively.


Rd
rd
S
Y
W
S
Y
W
200
116
97
66
31
22
14

CHAPTER IV.

CHARACTERIZATION OF RARE SPLICING PRODUCTS ASSOCIATED WITH THE *rd* ALLELE

IV. CHARACTERIZATION OF RARE SPLICING PRODUCTS ASSOCIATED WITH THE *rd* ALLELE

IV. A. INTRODUCTION

The previous chapter clearly shows that the *rd* allele is associated with a 5' splice site mutation in the gene for riboflavin binding protein. In the process of characterizing this defect a cDNA was cloned which was the result of incorrect splicing of the *rd* transcript (Fig. III. 2.). This splicing product is either very unstable or is produced inefficiently since it was not visualized on Northern blots of *rd* RNA (Fig. III. 1.). Via PCR it was possible to visualize, clone and sequence this product and show that this, the major splicing product in the *rd* , is the result of exon skipping induced by a G to A mutation in a 5' splice donor site in the gene for riboflavin binding protein.

Various researchers have studied the effects of G to A mutations and have used their results to draw conclusions about the splicing mechanism itself. At one time the requirement for a 5' G in splicing was thought to be limited to base pairing with U1 snRNA. However when Siliciano and Guthrie examined the efficiency of various mutant 5′ splice sites in the presence of compensating U1 snRNA they found that 5′ donor G to A mutations could not be rescued by compensating mutations in U1 snRNA (132), indicating a role for the 5′ G beyond U1 recognition and binding. The work of Aebi *et al.* (146) showed that the requirement for a 5' G is in the second step of the two step splicing pathway. When competing upstream 5' donor sites are removed in artificial constructs, a G to A mutation does not prevent the first step of splicing, 5' intron cleavage and lariat formation. Therefor the G would apparently have a role in the processes involved in 3' cleavage or exon ligation. This work is supported by the results of Newman and Norman(153) who have found that the conserved G may facilitate the correct positioning of U5 snRNP required for lariat processing in the second step of splicing.

In other cases where the 5′ splice donor sequence is perturbed the fidelity of the normal splicing pattern is disrupted (125, 126, 127, 128, 129). This often involves the activation of so-called cryptic splice sites. Cryptic splice sites resemble normal 5' splice donor sites and become activated in cases where the wild type splice donor site is mutated. In these cases the mutations usually reduce the complementarity between the 5' splice donor site and U1 snRNA. Cryptic splice sites can often be predicted by their complementarity to U1 snRNA (130). The coding region of the gene for ribBP contains one site which is highly complementary to U1 snRNA and could function as a cryptic splice site. The sequence GTAAGC begins at nucleotide 196 in the wild type cDNA for ribBP. The wild type 5' splice donor sequence in IVS2 is GTAAGT which matches the consensus for such sites exactly. The complementary region of U1 snRNA has the sequence CAUUCA (3' to 5'). Given the high level of complementarity between the sequence at nucleotide 196 and the U1 snRNA it seems likely that this site could be a cryptic 5' splice donor site. This possibility is investigated in this chapter.

Much of the characterization of the splicing pathway has come about via studies either *in vitro* using the SP6 system (146, 152), in cells which had been transiently transfected with artificial constructs (146) or in genetically manipulated yeast (124, 153). In the following study I have used an extension of the approach utilized in Chapter III to characterize rare splicing products of the mutant *rd* transcript. My approach differs from those previously mentioned in that these rare splicing products have been isolated from poly A^+ cytoplasmic RNAs transcribed from a native gene *in vivo*. Additionally, these other studies usually involve the use of specialized techniques such as primer extension (154) or S1 nuclease analysis (155) in the characterization of the splicing products. My approach was to clone and sequence rare transcripts which had been amplified by PCR. Using this approach I identified products of the gene for riboflavin binding protein which were the result of exon skipping, cryptic splice site activation and, surprisingly, correct splice site utilization in spite of the G to A mutation. The results of this investigation are presented here.

IV. B. RESULTS

IV. B. 1. PCR AMPLIFICATION OF RARE SPLICING PRODUCTS

In Chapter III, northern blot analysis of RNA from homozygous mutant and normal animals showed that very little message for ribBP could be detected in the *rd* hen (Fig III. 1.). However, as described in the Materials and Methods section, PCR can be used to amplify the protein coding region of this message from cDNA derived from both normal and, importantly, mutant liver mRNA. The reactions resulted in amplification of a 875 bp product in the *Rd* and a 775 bp product in the *rd* (Fig. III. 2.).The *rd* message contains a 100 nt deletion relative to the wild type. The *rd* deletion is in the coding region of the cDNA, removing nucleotides 140 - 239 from the *rd* message. These 100 nucleotides correspond exactly to one exon in the gene for riboflavin-binding protein.

The success of this approach in identifying a rare splicing product which could not be visualized on northern blots encouraged me to test for other rare splicing products from the *rd* transcript. As in Chapter III, cDNA was prepared using poly(A)$^+$-RNA isolated from laying hen liver tissue.

cDNA synthesis was primed with an excess of oligo(dT)$_{15}$ (Boehringer Mannheim 813 702) and the reaction was driven with Superscript™ Moloney Murine Leukemia Virus reverse transcriptase (Bethesda Research Laboratories, 8053SA) as described in Materials and Methods. cDNA produced in such a manner was used as template for PCR reactions. PCR was carried out on cDNA using primers directed against the wild-type ribBP cDNA (144). In this case two sets of primers were used to amplify the cDNA. The first set of primers, which we will call the 5' set, consists of ribBP Pos 1 and ribBP Neg 2 which have the sequences;

5′ CTC GAA TTC CAG AAG GAC AGC AAA AGA GGA 3′ and

5′ GGG AAT TCG TGC GGA GAA CAC CGG TAA 3′.

The underlined portions of the sequences correspond to the regions complementary to the respective target sequence. These primers contain 5′ *Eco*RI sites to facilitate cloning and manipulation of their products. The primer ribBP Pos 1 is directed against a site located in the 5' untranslated region of the ribBP cDNA, the primer ribBP Neg 2 is directed against a site immediately downstream from the 100 bp deletion (Fig. IV. 2.). The second set of primers used for amplification of cDNA which we will call the 3' set, consists of ribBP Pos 2 and ribBP Neg 1 which have the sequences;

5′ GGG AAT TCA TGA ATG CAC TCT GTA TTC T 3′ and

5′ GGG AAT TCG ATT TAT TGT GTT CTC AGA AGT GAG 3′,

respectively. Again the underlined portions of the sequences correspond to the regions complementary to the respective target sequence. These primers contain 5′ *Eco*RI sites to facilitate cloning and manipulation of their products. The primer ribBP Pos 2 is directed against a site immediately upstream from the 100 bp deletion and the primer ribBP Neg 1 is directed against a site in the 3' untranslated region of the ribBP cDNA.

The primers were used to amplify cDNA from both wild type and mutant livers. The first set of primers, the 5' set, has the capacity to amplify splicing products which resulted from splicing of any 5' donor site upstream from the 100 nt Exon2 to any 3' acceptor site upstream from the ribBP Neg2 primer annealing site, including the 3' splice acceptor site in IVS2. The second set of primers, the 3' set has the capacity to amplify any splicing product which resulted from splicing of any 5' donor site downstream from the ribBP Pos2 primer annealing site, including the 5' splice donor site in IVS1, to any 3' splice acceptor site upstream from the ribBP Neg1 primer annealing site. In the wild type these primers would be expected to result in the amplification of products which corresponded to portions of the ribBP cDNA, the 5' set amplifying the 5' portion of the cDNA and the 3' set amplifying the 3' portion. In fact when the 5' set was used to amplify cDNA from wild type hens, the major product migrated with an apparent size of 345 bp (Fig. IV. 1.). This corresponds with the predicted product of 345 bp. However a second, larger band was visible which had an apparent size of 450 bp. This band may correspond to a product which was occaisionally visible upon amplification of wild type cDNA with ribBP Pos1 and ribBP Neg1. Such an amplification results in a 875 product which corresponds to the full length cDNA for ribBP. However when this amplification was performed with low annealing temperatures a second, larger product was observed. It was originally thought that this was an artifact of the PCR reaction, possibly caused by the low annealing temperature. However, since a product with a similar size difference can be observed when amplifying with the 5' primer set this extra band may correspond to an incorrectly or alternatively spliced product in the wild type. When the 5' set of primers was used to amplify cDNA from hens homozygous for the *rd* allele another unexpected result was obtained. This amplification yielded a series of bands ranging from 450 to 114 bp in length (Fig. IV. 1.). The predicted size of the amplification product corresponding to one formed by skipping Exon 2 would be 245 bp. A 245 bp product was amplified but at the same time, products of 450, 345, 301 and 114 bp were also observed.

When the second set of primers, 3' primer set, was used to amplify the cDNA from wild type liver the predicted size of the amplification product would be 722 bp. This product would contain the 3' 722 bp of the ribBP cDNA. This product was observed. When the 3' primer set was used to amplify cDNA from hens homozygous for the *rd* allele the predicted product would be expected to be 100 bp smaller than the product of the wild type cDNA due to the exclusion of Exon2. In fact a 622 bp product was observed in the amplification of *rd* liver cDNA using the 3' primer set. No other products were observed in either the wild type or mutant cDNA amplifications when the 3' primer set was used (data not shown).

I.V. B. 2. CLONING AND CHARACTERIZATION OF RARE SPLICING PRODUCTS

In order to characterize the products of the amplifications described above, I cloned them into a vector which was suitable for sequencing. The PCR-amplified cDNAs were isolated and purified from low melting point agarose after electrophoresis. The fragments were ligated ligated directly into a pCR™ 1000 vector using the protocol supplied by the manufacturer (Invitrogen Corporation). These constructs were used to transform *E. coli* INVαF′. Transformants were selected, and plasmid DNA from these cells was digested with *Eco*RI and analysed by agarose gel electrophoresis. Sequencing of the cDNA clones was accomplished in both directions via the dideoxy-chain termination method using Sequenase™ (USB). Sequencing allowed unambiguous assignment of sizes and splicing patterns. The 5' product corresponding to the *Rd* cDNA for ribBP was cloned and had the predicted size of 345 bp. It was the result of normal splicing of Exon1-Exon2-Exon3 (Fig. IV. 3.). The 3' product corresponding to the *Rd* cDNA for ribBP was also cloned and sequenced and had the predicted size of 722 bp. This product was also the result of normal Exon1-Exon2-Exon3 splicing (Fig. IV. 3.).

When the products of the *rd* cDNA amplifications were cloned and sequenced a much more complicated picture emerged. The 5' product which corresponded to the *rd* cDNA described in Chapter III was cloned and had a predicted size of 245 bp. This product was the result of exon skipping due to the G to A mutation in the 5' splice donor site in IVS2. This event results in the removal of 100 bp from the *rd* ribBP cDNA. This product will be refered to as Δ100. A second 5' product was cloned from *rd* cDNA which had a size of 345 bp. Sequencing of this product shows that it is the result of correct splicing of Exon 2 to Exon3 in spite of the G to A mutation. In this case the resulting cDNA has the same sequence as the wild type cDNA and so it will be refered to as Δ0. A third 5' product was cloned and sequenced which had a size of 114 bp. Sequencing of this product shows that it is the result of skipping of not only Exon2 of the gene for ribBP but also a portion of the coding region upstream from Exon2 removing 231 bp from the cDNA for ribBP. This product will be refered to as Δ231. A fourth 5' product was cloned and sequenced which had a size of 301 bp. Analysis of this clone showed that it was the result of splicing of the normal 3' splice acceptor site in IVS2 to a site internal to Exon2. This site resembles the consensus for 5' splice donor sites. This site, a cryptic splice site, becomes activated when the normal 5' splice donor site is disrupted in the *rd*. Since the use of this cryptic splice site results in the removal of 44 bp from the cDNA for ribBP this splicing product will be refered to as Δ44. The locations of the splice sites and the splicing patterns which result in the Δ0, Δ44, Δ100 and Δ231 products are shown in Figs. IV. 2, 3, and 4.

The product of the *rd* amplifications using the 3' primer set was 622 bp. Sequencing showed that this product was again the result of skipping Exon2 and corresponded to the 3' portion of the cDNA presented in Chapter III.

IV. C. DISCUSSION

This study identifies four rare splicing products resulting from the *rd* mutation in the gene for riboflavin binding protein. The most abundant of these splicing products, Δ100, is the product formed by skipping of Exon2 (Fig. IV. 5.). This is the product which was characterized in Chapter III. A second product, Δ44, is the result of cryptic splice site usage (Fig IV. 6.). A cryptic 5' splice donor site, containing the sequence GTAAGC, lies in the coding region of the gene for riboflavin binding protein (Fig. IV. 2.). This site is used when the wild type 5' splice donor site is disrupted due to the G to A mutation in the *rd*. Based on the sequence complementarity to the U1 snRNA the utilization of this splice-site and the formation of the Δ44 splicing product was predicted at the onset of this study. A third splicing product, Δ231, results from skipping of not one but two exons upstream from the *rd* mutation (Fig. IV. 2.). A fourth and entirely unexpected product, Δ0, results from maintainance of the normal splicing pattern in spite of the G to A mutation.

Exon skipping as a result of 5' G to A mutations in a splice donor site has been observed previously. It is known to be the cause of other genetic diseases besides avian riboflavinuria (156). For example exon skipping as a result of a G to A mutation in the gene for human ß-globin is the cause of one form of $ß^{o}$-thalassemia (151). However, to my knowledge skipping of more than one exon as a result of such a mutation has not been reported. This may be due to the relative insensitivity of primer extension analysis when compared to the approach used here in the characterization of the rare splicing products. It should be emphasized that the majority of pre-mRNA would be expected to be processed normally until the second step of splicing, resulting in the accumulation of dead-end lariat intermediates. These intermediates are not detected with the approach used in this study. The PCR approach is however capable of characterizing splicing products which are not visible upon Northern analysis. Surprizingly, in spite of the *rd* mutation, a small amount of normally spliced ribBP cDNA, Δ0, can be detected in hens homozygous for the *rd* allele. Once again this is an illustration of the sensitivity of this technique as correctly spliced products are not usually observed upon primer extension analysis of G to A mutant containing transcripts (127, 146, 151, 157). The level of correctly spliced message must be very low as it is not visible upon Northern analysis (Fig. III. 1.) nor is there any detectable ribBP in hens homozygous for the *rd* allele (Fig. III. 7.).

Exon skipping is often seen *in vivo* where it occurs naturally as a method of controlling gene expression, often involving the choice between exon skipping or inclusion (158, 159). This process involves both *trans*-acting nuclear factors and *cis*-acting sequence elements, often other than those normally associated with the regulation of the splicing mechanism. For example the tissue specific expression of ß-tropomyosin pre-mRNA involves the exclusion or inclusion of mutually exclusive exons. This process is effected by the splicing factor SF2, which promotes exon inclusion, and by the factor hnRNP A1 (heterologous nuclear ribonucleoprotein A1), which promotes exon skipping (160). Current models indicate that several elements, the 5' splice donor site, the 3' acceptor site, the branch point, the polypyrimadine tract, the length of the exon and the length of the downstream intron as well as the *trans* acting splicing factors all cooperate to determine the selection of exons in alternate splicing pathways (161). The splicing pattern of the ribBP pre-mRNA may exhibit a small degree of tissue specificity (Fig. IV. 1.). The Δ44 and Δ0 products are more abundant in RNA isolated from the oviduct than in liver RNA. It is thought that tissue specificity of alternative splice site selection is modulated by *cis*-acting splicing factors such as hnRNP A1 and SF2 (117, 160). These or other similar factors may play a role in the differential formation of these products in these tissues.

The currently accepted splicing model which invokes the binding of U1 to the 5' splice donor site followed by U2 binding at the branch point implies that the intron is the unit of recognition in splicing. Such a model would involves pairwise recognition of the splice sites as a result of a scanning mechanism which moves along the newly synthesised message in the 5' to 3' direction (162). This is consistant with the "first come, first served" model in which pre-mRNA synthesis is coupled to the formation of "committed" pre-splicing complexes (146). In this case the fidelity of splicing 5' to a splice site mutation is maintained by the polarity in splice site recognition. However no such polarity in splice site recognition has been found (163). The results presented in this study show clearly that the splicing pattern 5' to the splice donor site mutation is not maintained and as such the "first come, first served" model does not strictly apply. Results similar to these have lead others to suggest that the exon rather than the intron is the recognised unit in splicing (164). This suggestion is thought to be supported by studies which describe the effects of terminal 5' splice donor sites on polyadenylation. Normally 3' terminal exons do not terminate with 5' splice donor sites. However when 5' splice sites are artificially inserted into terminal exons polyadenylation is inhibited. The authors conclude that they have evidence for 5' to 3' splice site recognition across exons rather than introns (164). They do not however, describe how this idea could be reconciled with the current model for the splicing mechanism. It is clear from the results of the current study that the outcome of a sequence mediated disruption of the splicing pathway is not entirely predictable regardless of the model used to describe splicing. Apparently there is even some variability in the stringency of certain "requirements" of the normal splicing pathway. For this reason it is all the more important to have at ones disposal a variety of techniques with which to analyse splicing. The technique used in this study provides one with unambiguous information which facilitates the determination of splice junctions. As cloning and sequencing of PCR products becomes more routine, modified versions of this technique could become an alternative to primer extension and other types of analysis.

Figure IV. 1.

PCR amplification of rare splicing products associated with the *rd* allele. Poly-A RNA was isolated from liver (L) or oviduct (O) of both homozygous wild type (*Rd*) and mutant (*rd*) hen. RT-PCR was performed as described in Materials and Methods (Chapter II) using the 5' primer set. Aliquots of 10 µl from a 100 µl reaction mixture were subjected to electrophoresis on a 1.4% agarose gel. The major band in the *Rd* samples cooresponds to the correctly spliced ribBP message. The minor bands in the *rd* samples coorespond to alternately spliced products which are discussed in the text.

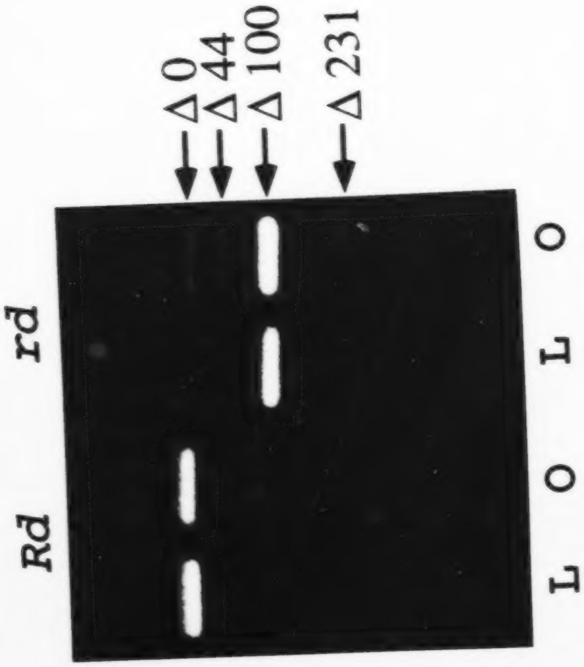

Rd
rd
L
O
L
O
Δ 0
Δ 44
Δ 100
Δ 231

Figure IV. 2.

Location of splice junctions of rare splicing products associated with the *rd* allele in the nucleotide sequence of the wild type cDNA for riboflavin binding protein. Splice junctions are shown for the Δ231, Δ100, Δ44 and Δ0 splicing products. The 100 nucleotides missing from the *rd* cDNA clone described in Chapter III (Δ100) are indicated in lower case (140 - 239). The underlined regions indicate the positions of the 5' primer set as described in the text. Numbering of nucleotides begins with the initiation codon. The deduced amino acid sequence is shown below the nucleotide sequence. Numbering of amino acids refers to the amino terminus of the mature wildtype protein.

```
                                                     1   Δ231                                                       50
             |           |           |               |     \     |           |           |           |               |
CCC AGA AGG ACA GCA AAA GAG GAA AAG AAG GGA ACA ACA ATG CTG AGG TTT GCC ATC ACC CTC TTT GCT GTC ATC ACA TCA TCT ACC TGC
                                                    Met Leu Arg Phe Ala Ile Thr Leu Phe Ala Val Ile Thr Ser Ser Thr Cys
                                                   -17
                                                                100                                           Δ100
         |               |           |           |               |           |           |               |          \|
CAG CAG TAT GGA TGT CTG GAA GGG GAC ACC CAC AAA GCG AAT CCA AGT CCT GAG CCA AAC ATG CAT GAA TGC ACT CTG TAT TCT GAA Tct
Gln Gln Tyr Gly Cys Leu Glu Gly Asp Thr His Lys Ala Asn Pro Ser Pro Glu Pro Asn Met His Glu Cys Thr Leu Tyr Ser Glu Ser
 1                                   10                                      20                                      30
        150                                                         Δ44     200
         |               |           |           |               |     \     |           |               |           |
tcc tgt tgc tat gca aac ttc aca gag caa ttg gct cat tcc cca ata att aaa gta agc aac agc tac tgg aac aga tgt ggg cag ctc
Ser Cys Cys Tyr Ala Asn Phe Thr Glu Gln Leu Ala His Ser Pro Ile Ile Lys Val Ser Asn Ser Tyr Trp Asn Arg Cys Gly Gln Leu
                                     40                                      50                                      60
     Δ0                 250                                                                 300
        \|               |           |           |               |           |           |               |           |
agt aaa tcC TGT GAA GAT TTC ACA AAG AAA ATC GAG TGC TTT TAC CGG TGT TCT CCG CAC GCT GCT CGC TGG ATC GAT CCC AGA TAT ACT
Ser Lys Ser Cys Glu Asp Phe Thr Lys Lys Ile Glu Cys Phe Thr Arg Cys Ser Pro His Ala Ala Arg Trp Ile Asp Pro Arg Tyr Thr
                                     70                                      80                                      90
                                    350                                                                     400
         |               |           |           |               |           |           |               |           |
GCT GCT ATT CAG TCT GTT CCA CTG TGT CAG AGC TTC TGT GAT GAC TGG TAT GAA GCC TGC AAA GAT GAT TCC ATT TGT GCT CAT AAC TGG
Ala Ala Ile Gln Ser Val Pro Leu Cys Gln Ser Phe Cys Asp Asp Trp Pyr Glu Ala Cys Lys Asp Asp Ser Ile Cys Ala His Asn Trp
                                    100                                     110                                     120
                                                    450                                                             500
         |               |           |           |               |           |           |               |           |
CTG ACG GAC TGG GAA CGG GAT GAA AGT GGA GAA AAC CAC TGT AAG AGT AAA TGC GTA CCA TAC AGT GAG ATG TAT GCA AAT GGG ACC GAC
Leu Thr Asp Trp Glu Arg Asp Glu Ser Gly Glu Asn His Cys Lys Ser Lys Cys Val Pro Tyr Ser Glu Met Tyr Ala Asn Gly Thr Asp
                                    130                                     140                                     150
                                                                550
         |               |           |           |               |           |           |               |           |
ATG TGC CAG AGT ATG TGG GGG GAA TCC TTT AAG GTG AGC GAA TCC TCC TGC CTC TGC TTG CAA ATG AAC AAG AAG GAC ATG GTG GCA ATC
Met Cys Gln Ser Met Trp Gly Glu Ser Phe Lys Val Ser Gly Ser Ser Cys Leu Cys Leu Gln Met Asn Lys Lys Asp Met Val Ala Ile
                                    160                                     170                                     180
        600                                                                 650
         |               |           |           |               |           |           |               |           |
AAG CAC CTC CTC TCC GAA AGC TCA GAG GAA AGC TCC AGT ATG AGC AGC AGT GAG GAG CAC GCC TGC CAA AAG AAA CTC CTG AAG TTT GAG
Lys His Leu Leu Ser Glu Ser Ser Glu Glu Ser Ser Ser Met Ser Ser Ser Glu Glu His Ala Cys Gln Lys Lys Leu Leu Lys Phe Glu
                                    190                                     200                                     210
                        700                                                                 750
         |               |           |           |               |           |           |               |           |
GCA CTG CAG CAA GAG GAA GGG GAA GAG AGA AGA TGA ATT TTG GTG GAT GAA TAT CAG GAG GAG AGG AAT CAT TGT GGA GGT TGT GCT CGG
Ala Leu Gln Gln Glu Glu Gly Glu Glu Arg Arg
                                    220
                                    800
         |               |           |           |           |
GGC ATC ACA GCA GCC TGT CTT ATC CCT CAC TTC TGA GAA CAC AAT AAA TC
```

Figure IV. 3.

Schematic diagram of splicing patterns associated with the *rd* allele. The wild type splicing pattern is shown at the top of the Fig. The lower portion of the Fig. diagrams the splicing patterns resulting in the products characterized in this study. The *rd* G to A mutation is in the 5' splice donor of IVS2. The major product, Δ100, is formed by skipping Exon2. The Δ0 product is formed by utilization of the normal splicing pathway in spite of the 5' G to A mutation. The cryptic splice site which is utilized in the production of the Δ44 product is internal to Exon2. The Δ231 product is formed by skipping both Exon1 and Exon2, splicing Exon3 to the Exon upstream from Exon1 (not indicated in the Fig.).

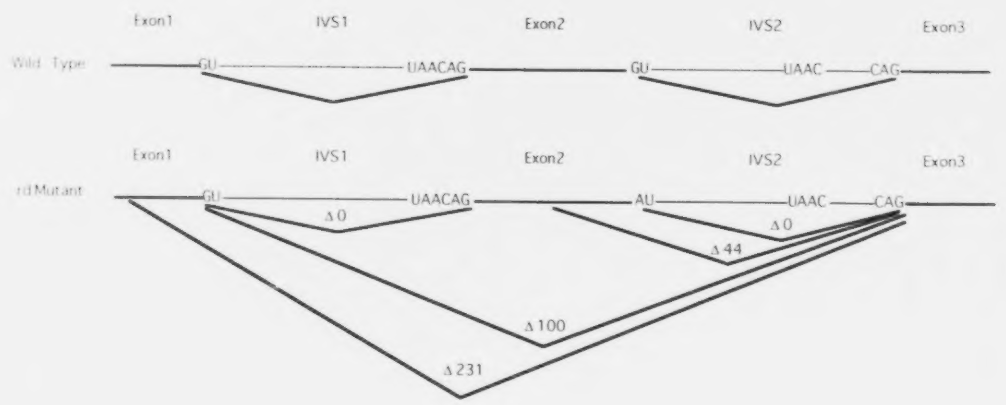

Exon1
IVS1
Exon2
IVS2
Exon3
Wild Type
GU
UAACAG
GU
UAAC
CAG
Exon1
IVS1
Exon2
IVS2
Exon3
rd Mutant
GU
UAACAG
AU
UAAC
CAG
Δ0
Δ0
Δ44
Δ100
Δ231

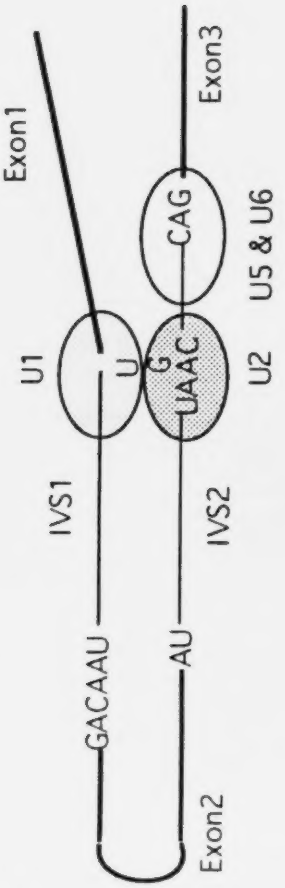
Figure IV. 4.

A summary of the products of the *rd* mutation. The splicing products characterized in this study are listed along with their sizes, the splice junctions and the mechanisms which lead to their formation.

Product	Size(bp)	Spliced Sequence	Mechanism
Δ0	345	CAG TAA ATC / CTG TGA AGA	Normal Splicing
Δ44	301	ATA ATT AAA / CTG TGA AGA	Cryptic Splicing
Δ100	245	ATT CTG AAT / CTG TGA AGA	Exon Skipping
Δ231	114	AAT GCT GAG / CTG TGA AGA	Exon Skipping

Figure IV. 5.

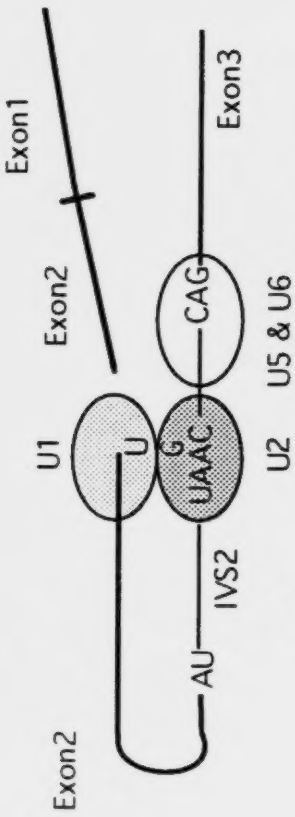
The mechanism leading to exon skipping. Because of a reduced complementarity between the 5' splice donor site and U1 snRNA, the U1 SNRP is slow to associate with the mutant 5' splice donor site. As a result the U2 SNRP at the branch point sequence interacts with the U1 particle associated with the upstream intron. The two step splicing reaction preceeds and Exon1 is ligated to Exon3.


Exon1
U1
U
G
UAAC
U2
CAG
U5 & U6
Exon3
IVS1
IVS2
GACAAU
AU
Exon2

Exon Skipping

Figure IV. 6.

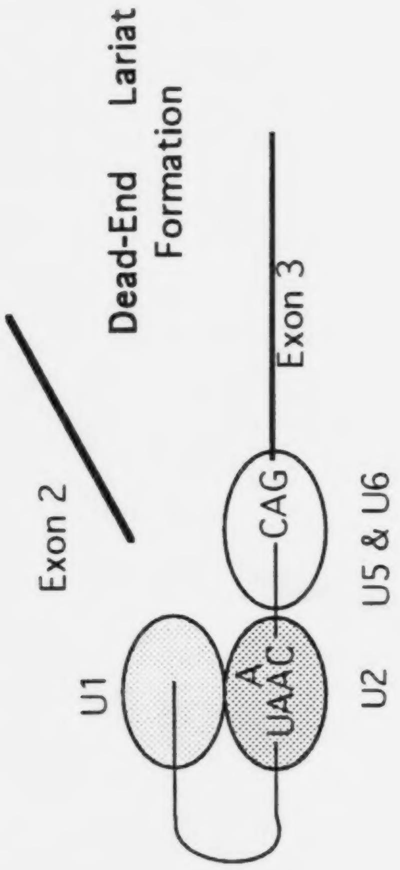
Mechanism leading to cryptic splice site activation. Perturbations in the wild type 5' splice donor site often lead to the activation of cryptic splice sites. These sites resemble the normal 5' splice donor sequence and are complementary to the U1 snRNA. One such site, internal to Exon2, becomes active in the *rd* and leads to the formation of the Δ44 splicing product.


Exon2
AU
IVS2
U1
U
G
UAAC
U2
Exon2
CAG
U5 & U6
Exon1
Exon3

Cryptic Splice Site Activation

Figure IV. 7.

Mechanism leading to dead end lariat formation. In spite of the 5' G to A mutation the U1 snRNA is capable of base pairing with the mutant splice donor site. The first step of splicing, 5' Exon cleavage and lariat formation, preceeds. However due to a requirement for the 5' G in the second step of splicing, 3' cleavage at the splice acceptor site and exon ligation, is blocked. The result is the formation of dead end lariat intermediates. Dead end lariats are not detected by the PCR analysis method used in this study.


Dead-End Lariat Formation
Exon 2
Exon 3
U1
A
UAAC
CAG
U2
U5 & U6

CHAPTER V.

ribBP BINDING TO MEMBERS OF THE AVIAN LIPOPROTEIN RECEPTOR FAMILY

V. ribBP BINDING TO MEMBERS OF THE AVIAN LIPOPROTEIN RECEPTOR FAMILY

V. A. INTRODUCTION

The uptake mechanisms of many of the yolk precursors, including the vitamin binding proteins, have yet to be elucidated. The purpose of this study is to characterize the mechanism by which the vitamin binding protein, riboflavin binding protein (ribBP) is taken up and sequestered by the growing oocyte.

Riboflavin binding protein is taken up specifically against an increasing concentration gradient. This in itself is thought to be evidence for receptor mediated uptake. Present in the serum at 0.064 mg/ml, it is concentrated as much as nine fold in the egg yolk at 0.56 mg/ml (11). This is similar to the relative concentrations of VTG in serum and egg yolk. The amount of VTG in the egg yolk is roughly six fold the concentration found in serum. Upon deposition in the oocyte ribBP undergoes a specific, protein mediated, proteolytic cleavage removing the carboxy-terminal 11 or 13 amino acids (7). This processing is analogous to the oocytic cleavage of both VTG and apolipoprotein B by the enzyme cathepsin D (60). Post endocytotic processing may be a motif common to the yolk precursors or may imply a similar uptake mechanism.

I undertook to determine the mechanism of ribBP uptake in the laying hen using certain techniques which had been developed in our laboratory. These techniques had been responsible for the successfull elucidation of the mechanism of VTG and VLDL uptake in the laying hen. One of these techniques is ligand blotting with oocyte membrane extracts. I purified sribBP and used it as a ligand in ligand blotting experiments. These experiments showed that purified ribBP had some affinity for the previously characterized

95 kDa oocyte membrane receptor for VLDL and VTG. In some experiments ribBP could also be seen to bind to the oocyte LRP. In order to quantitate the affinity of ribBP for these lipoprotein receptors I developed a binding assay which measured ^{125}I-labeled sribBP binding to crude oocyte membrane extracts. Using this assay it was possible to demonstrate saturable, competable binding of sribBP to oocyte membrane extracts. I also developed an antibody against ribBP and using this antibody visualized native sribBP binding to oocyte membrane extracts. These results are presented here.

V. B. RESULTS

V. B. 1. BINDING OF ^{125}I-LABELED SERUM ribBP TO OOCYTE MEMBRANE EXTRACTS

RibBP was purified using a method derived from a previously published procedure (133). This method was followed by FPLC on a MONO-Q ion exchange column (Material and Methods, Chapter II). The resulting purified serum, egg yolk and egg white ribBPs are shown in Fig. V. 1. FPLC pure sribBP was radiolabeled and used as a ligand in ligand bloting experiments against detergent solubilized oocyte membrane proteins. The membrane proteins were resolved by SDS-polyacrylamide electrophoresis and transfered to nitrocellulose membranes. Purified ^{125}I-labled sribBP was shown to bind to a protein present in oocyte membranes (Fig. V. 2.). This was the first demonstration of ribBP binding to a specific oocyte membrane protein. An essential characteristic of any receptor-ligand interaction is that it be saturable. ^{125}I-sribBP binding to the membrane protein on ligand blots can be competed with excess unlabeled sribBP, indicating a finite number of binding sites (Fig. V. 2.). Fig. V. 2. shows that competition is essentially complete with as little as 20 fold molar excess of unlabled sribBP. Also shown in this figure is the abolition of sribBP binding by suramin. Suramin is a heterocyclic polyanionic compound known to inhibit receptor ligand interactions (165, 166). In particular it has been shown to abolish the binding of VLDL and VTG to the oocyte membrane receptor (1, 2).

The protein which binds sribBP migrates slightly below the 97 kDa standard. The oocyte receptor for VLDL and VTG has a molecular weight of 95 kDa (6). Parallel experiments with ^{125}I-labeled sribBP and VLDL clearly show the comigration of the proteins binding these two ligands (Fig. V. III.). To test the intriguing possibility that sribBP may be binding to the 95 kDa receptor for VLDL and VTG, I attempted to inhibit the binding of ^{125}I-labeled sribBP to oocyte membranes with F_{ab} fragments prepared from antibodies directed against the bovine LDL receptor. These F_{ab} fragments had previously been shown to inhibit the binding of $^{125-}$I-labeled VLDL to the 95 kDa receptor. Fig. V. 4. shows that the binding of ^{125}I-labeled sribBP to oocyte membranes is inhibited by the addition of F_{ab} fragments from antibodies dirrected against the bovine LDL receptor. This inhibition begins at concentrations as low as 10 µg/ml in the incubation buffer and is complete by 100 µg/ml. Further evidence to support the hypothesis that sribBP binds to the 95 kDa receptor is shown in Fig. V. 5. In this case ^{125}I-labeled sribBP binding to the 95 kDa receptor is competed by the addition of 100 fold molar exess of laying hen VLDL. Binding is completely abolished in the presence of 100 fold molar excess VLDL.

V. B. 2. QUANTITATIVE BINDING ANALYSIS

The conditions required to visualize binding of sribBP in ligand blotting experiments, large amounts of labeled protein combined with long exposure times, were thought to be indicative of a weak interaction with the 95 kDa receptor. In order to compare qualitatively the binding of sribBP and the binding of VLDL to the 95 kDa receptor, an attempt was made to compete the binding of ^{125}I-labeled laying hen VLDL to the 95 kDa receptor with unlabeled ribBPs. RibBPs were purified from laying hen serum, egg yolk and egg white . Addition of 100 or 200 fold molar excess of serum , egg yolk or egg white ribBP to incubations with ^{125}I-labeled VLDL had no appreciable effect on VLDL binding to the 95 kDa receptor (Fig. V. 6.). This result could be interpreted in two ways. Firstly, ribBP may have a much lower affinity for the 95 kDa receptor than does VLDL. In this case it would be unable to displace bound VLDL. The second explanation would involve a model where the 95 kDa receptor had multiple ligand binding sites. The binding of ribBP to one of these binding sites would not necessarily exclude the binding of VLDL. However Fig. V. 5. shows that VLDL is capable of competing for sribBP binding to the 95 kDa receptor. The second explanation therefor seems unlikely. In this case one would have to accept the likelyhood that the binding affinity of sribBP for the 95 kDa receptor was significantly lower than that of VLDL. In order to further characterize this phenomena a binding assay for ribBP binding to oocyte membrane extracts was developed.

The 95 kDa receptor had been partially characterized by the use of a solid phase filtration binding assay. This assay had enabled Drs. Barber and Stifani to quantitate the interactions between the receptor and the ligands VLDL and VTG (1, 2). This assay involved the separation of receptor-ligand complexes from free ligand by filtration through cellulose acetate filters. When the binding of ribBP to ooctye membrane extracts was measured using this technique the results were inconsistant (data not shown). In order to measure ribBP binding to oocyte membrane extracts an assay was developed which relied on centrifugal force to separate receptor-ligand complexes from free ligand. In this case oocyte membrane pellets were resuspended in buffer containing the detergent octyl-glucoside. The membranes were incubated with increasing concentrations of ^{125}-I-labeled sribBP for a period of one and a half hours. After this time the receptor-bound ligand was precipitated by centrifugation in a Beckman Airfuge. The free ligand remains soluble under the conditions of the assay. The receptor-ligand pellet was then washed and the bound ligand quantitated by counting in a gamma counter. Using this approach it is possible to measure saturable binding of ^{125}I-labeled sribBP to oocyte membranes (Fig. V. 7.). It was also possible to demonstrate that the binding of sribBP to oocyte membrane extracts is Ca^{2+} dependant. The addition of 20 mM EDTA to the incubations results in the linear, non-specific binding profile shown in the saturation curve (Fig. V. 7.). This corroborated earlir results where it was found that there is a Ca^{2+} requirement for sribBP binding in ligand blotting experiments (data not shown). Ca^{2+} is required for the binding of all of the known ligands of the the avian lipoprotein receptor family. By subtracting the non-specific binding measured in the presence of EDTA from the total binding it was possible to calculate the specific binding component of the total ligand binding (Fig. V. 7.). This data was used to perform Scatchard analysis of sribBP binding to oocyte membrane extracts (Fig. V. 8.) (167). This analysis revealed a K_d of 1.3 μM for sribBP binding to oocyte membrane extracts. The K_d for laying hen VLDL binding to purified 95 kDa receptor is 6.8 nM (168). The K_d for VTG binding to the same receptor is 6.7 nM. These results clearly show that sribBP has an affinity for the oocyte membrane receptor that is several orders of magnitude lower than that of VLDL or VTG.

Competition experiments quantitated the binding of ^{125}I-labeled sribBP in the presence of excess unlabeled sribBP. These experiments showed that the binding of ^{125}I-labeled sribBP to oocyte membrane extracts could be competed by the addition of unlabeled sribBP (Fig. V. 9.). This corroborates the results of the experiment shown in Fig. V. 2.

V. B. 3. LIPOPROTEIN RECEPTOR BINDING OF ribBP IN ESTROGEN TREATED ROOSTER SERUM

Given the weak interaction between ribBP and the 95 kDa receptor an alternate approach was used to visualize ribBP binding to oocyte membrane proteins. Again membrane extracts were resolved by SDS-polyacrylamide electrophoresis and transfered to nitrocellulose membranes. These membranes were incubated in either the presence of whole estrogenized rooster serum, which contains large amounts of ribBP, or in control rooster serum which contains no ribBP. The rationale for this experiment was that there may be some other factor, present in serum, which facilitates the binding of ribBP to a putative receptor in oocyte membranes. In the process of purification this factor would be removed and binding of purified ribBP to oocyte membranes would not reflect the situation *in vivo*. However native ribBP binding to oocyte membranes can be observed using an antibody directed against ribBP. Commercially available egg white ribBP (Sigma) was injected into a rabbit and the resulting polyclonal antibody was purified on protein A sepharose. This antibody was shown to recognize the serum, egg yolk and egg white isoforms of ribBP. Fig. V. 10. shows the results of a ligand blot where native sribBP bound to oocyte membrane proteins has been visualized with this antibody. The results show that ribBP, present in estrogenised rooster serum binds to two proteins present in oocyte membrane extracts. These proteins have the same molecular weights as the 95 kDa receptor and the oocyte LRP. Further evidence that under these conditions, ribBP binds specifically to the 95 kDa receptor is shown in the accompanying blots using oocyte membrane extracts from R/O hens. R/O hens are from a strain of chickens which lack a functional 95 kDa oocyte receptor (145, 169, 170). These hens are unable to transport VLDL and VTG into the oocyte and as a result yolk precursors accumulate in the plasma. The high levels of VLDL induce atherosclerosis and these hens often die of myocardial infarction. Because they lack a functional 95 kDa receptor, oocyte membranes extracts isolated from R/O hens do not bind VLDL or VTG. Fig. V. 10. shows that R/O membranes do not bind sribBP at the 95 kDa band. This is genetic evidence in support of the hypothesis that sribBP enters the oocyte via the 95 kDa receptor. Fig. V. 10. also shows binding of native sribBP to the oocyte LRP. R/O hens do have functional LRP and sribBP can be seen to bind to the LRP in oocyte membrane extracts from R/O hens. The laying hen expresses at least two other members of the LDL receptor gene family. Chickens have an LDL receptor analog with an apparent M_r of 130 kDa (114). This receptor binds apoB containing lipoproteins and is thought to be involved in the regulation of somatic cell cholesterol homeostasis. As well, the laying hen expresses a somatic cell specific LRP (112, 113). These two proteins are present in extracts prepared from chicken liver membranes. Fig. V. 10. shows that when these membrane extracts are probed for ribBP binding activity no such activity can be detected. These results indicate that the ribBP binding attributes of the avian LDL receptor family may be confined to the oocyte specific members of that family.

V. C. DISCUSSION

The experiments described in this chapter are the first to demonstrate ribBP binding to specific oocyte membrane proteins. There is a considerable body of evidence to suggest that ribBP is taken up by receptor mediated endocytosis. Free riboflavin is not taken up by the oocyte, therefor the uptake of riboflavin must be mediated by the structural features of ribBP. Although some yolk precursors are synthesised in the follicle, all of the ribBP found in the egg yolk is hepatically derived. *In vivo* studies have shown that radiolabeled ribBPs are taken up by the oocyte in laying hens (50). The directed uptake of ribBP results in an increase in yribBP concentration to as much as nine fold that of sribBP (11). In spite of the evidence suggesting receptor mediated uptake, attempts to identify a receptor for ribBP have been inconclusive (171). Here I have used ligand blotting tequniques to demonstrate the binding of ribBP to both the 95 kDa oocyte membrane receptor and the oocyte LRP.

A prerequisite for ligand binding experiments is the preparation of pure ligand. I modified an established technique to purify ribBPs from serum, egg yolk and egg white (133). The resulting ribBP was subjected to FPLC on a Mono-Q ion-exchange column. The chosen gradient conditions included a change in both counter-ion concentration and pH. This treatment resulted in the preparation of pure ribBP which were subjected to analysis by SDS-PAGE (Fig. V. 1.). RibBPs are phosphoglycoproteins which appear as diffuse bands on SDS-PAGE as a result of heterogeneities in both the composition of the sugar side chains and the pattern of phosphorylation of the eight serines clustered near the carboxy terminus (50, 54, 172, 173). Upon reduction, ribBPs decrease in electrophoretic mobility as a result of relaxation and unfolding of their normally highly condensed structure (Fig. V. 1.). It is unlikely that reduction of any potential co-purifying contaminant protein would result in identical electrophoretic shifts. Therefor the electrophoretic analysis shown in Fig. V. 1. clearly demonstrates the purity of the ribBP preparations which were used as ligands in the ligand blotting experiments and binding assays.

The ligand blotting experiments demonstrate that sribBP binds to the 95 kDa oocyte membrane receptor as well as the oocyte LRP. This binding is specific and can be competed with excess unlabeled sribBP (Figs. V. 2, V. 9.). The binding of sribBP to the 95 kDa receptor shares several features with apoB mediated VLDL binding to the same receptor. Both sribBP and VLDL binding require calcium (1). Both interactions are abolished by the heterocyclic, polyanionic compound suramin (Fig. V. 2.) (1). As well, sribBP binding to the 95 kDa receptor can be competed by VLDL (Fig. V. 5.). Antibodies which abolish VLDL binding to the 95 kDa receptor also abolish the binding of sribBP (Fig. V. 4.). As well as apoB containing lipoproteins, the 95 kDa receptor has been shown to bind VTG, Ca^{2+}, α_2-macroglobulin and the mammalian ligand apolipoprotein E (41). The binding of these various ligands to the 95 kDa receptor is thought to be mediated by the type A ligand binding or SDE repeats. These negatively charged SDE repeats are thought to interact cooperatively with positively charged regions on the ligands. A positively charged region of human apo B has been implicated in binding to the LDL receptor (23).This region is located between residues 3359-3368 (Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys-Leu). The vitellogenins contain two clusters of positively charged and hydrophobic residues located in the lipovitellin-I portion of VTG at residues 1079-1084 (Lys-Leu-Lys-Arg-Ile-Leu) and 493-498 (Leu-Lys-Arg-Ile-Leu-Lys). These regions resemble a region in the putative receptor binding site of apo E between residues 143-150 (Lys-Leu-Arg-Lys-Arg-Leu-Leu-Arg) (41). A positively charged and hydrophobic sequence (Glu-Lys-Lys-Leu-Leu-Lys) lies near the carboxy-terminus of sribBP at residues 203-208. These residues may mediate the binding of ribBP to the avian lipoprotein receptor family. Interestingly, the post-endocytotic processing of yribBP involves cleavage at either residue 206 or 208. In the later case this would remove two of the six residues in this putative receptor binding region.

In light of the observation that sribBP has binding affinity for both the 95 kDa oocyte membrane receptor and the oocyte LRP, one might expect to observe a biphasic curve upon Scatchard analysis of the quantitative binding data. Although the curve shown in Fig. V. 8. could be interpreted as being biphasic, and therefor indicative of two binding sites with different affinities for the ligand ribBP, this feature was not shared with the curves obtained in similar experiments. As well, Scatchard analysis of the binding affinity of VTG and VLDL for crude oocyte membrane extracts does not normally detect the differential binding affinities of the 95 kDa receptor and the oocyte LRP. For this reason, and because the affinity of the oocyte LRP for the ligand ribBP appeared to be much lower than that of the 95 kDa receptor, the results obtained in the quantitative binding assay were taken to be representative mostly, if not entirely, of the binding affinity of the 95 kDa receptor for sribBP. These results indicate that the binding of ribBP to oocyte membrane proteins is saturable, that there is a finite number of binding sites. However this assay also yields a very high K_d for ribBP binding to oocyte membranes (1.3 μM), therefor the affinity of ribBP for oocyte membrane proteins is low. Since the affinity of pure ribBP for oocyte membranes is not of the magnitude required to facilitate oocytic uptake, an alternate approach was used to visualize ribBP binding to oocyte membrane proteins. In this instance whole estrogenized rooster serum, containing large amounts of ribBP, was incubated with oocyte membrane extracts. The bound ribBP was visualized with an antibody against egg white ribBP. This experiment yielded very encouraging results. RibBP was shown to bind to both the 95 kDa receptor and to the oocyte LRP. If the binding of native ribBP in serum is stronger than that of purified ribBP it could be due to a number of factors. Either (i) radiolabelling ribBP interferes with receptor binding or (ii) the purification process destroys some feature of ribBP which is required for optimal binding or (iii) the purification process removes some other factor, present in serum, which facilitates the binding of ribBP to oocyte membrane receptors. In either case the binding of purified ribBP to oocyte membranes would not reflect the situation *in vivo*. The idea that ribBP may be taken up in the oocyte in concert with other yolk precursors is not entirely new. White and Merril (43, 174) have put forward the hypothesis that ribBP could be deposited in the oocyte as part of a complex formed between ribBP and other yolk precursors. Specifically they have suggested that ribBP may associate with VTG, another phosphoglycoprotein precursor, via calcium phosphate cross-bridges. In this case ribBP would be taken up as part of an aggregate "parasitizing" the VTG receptor system. Here I have shown that ribBP alone has some affinity for the oocyte membrane lipoprotein receptors and that native ribBP in the presence of other yolk precursors binds strongly to the 95 kDa receptor and to the oocyte LRP. The interactions between ribBP and other yolk precursors, in particular VTG, will be described in detail in the next chapter.

Figure V. 1.

SDS-polyacrylamide gel electrophoresis of chicken ribBPs. RibBPs were purified as described in the Materials and Methods section (Chapter II) from egg yolk (Y), laying hen serum (S) and egg white. All samples contained 20 µg of protein. Samples were analyzed on a 4.5-18% polyacrylamide gradient gel. Reduced samples were heated to 90° C for five min in the presence of 10 mM dithiothreitol. The proteins were stained with coomasie blue.

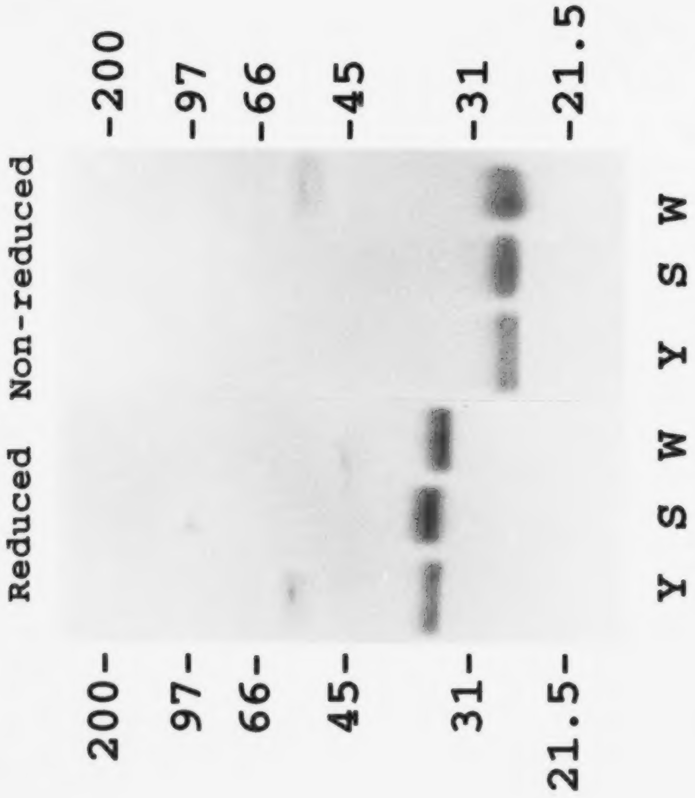

Reduced
Non-reduced
200-
97-
66-
45-
31-
21.5-
-200
-97
-66
-45
-31
-21.5
Y S W
Y S W

Figure V. 2.

Ligand blotting of radiolabelled serum ribBP. Oocyte membrane proteins (30 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods (Chapter II). Radiolabeled serum ribBP (1.25 μg/ml; specific activity 8 kcpm/ng) was incubated with the nitrocellulose in 20 mM Tris-HCl, 90 mM NaCl, 2 mM $CaCl_2$ pH 7.4 for 4 hr. Labeled serum ribBP binding is competed with 1, 2, 5, 10 and 20 fold molar excess of unlabeled serum ribBP. One incubation was in the presence of 10 mg/ml suramin. Autoradiography was for 48 hr.

I^{125} sRibBP Binding to Oocyte Membrane Extracts

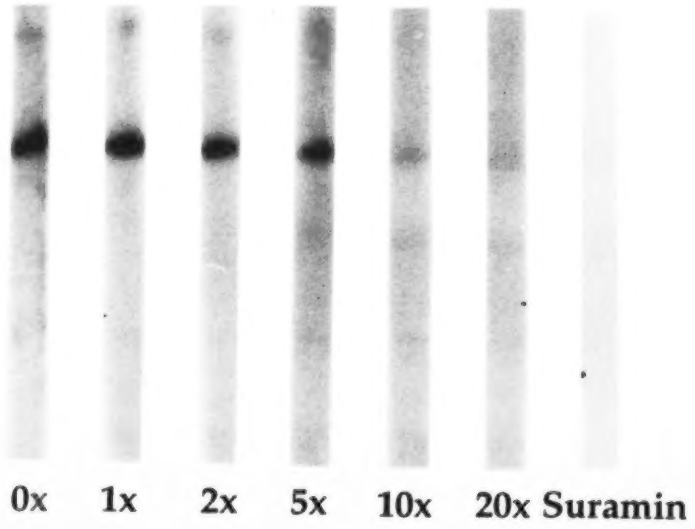

0x
1x
2x
5x
10x
20x
Suramin

Figure V. 3.

Ligand blotting analysis of the 95 kDa oocyte membrane receptor. Oocyte membrane proteins (30 µg/lane) were separated by SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods (Chapter II). Radiolabeled serum ribBP (1.25 µg/ml; specific activity 8 kcpm/ng) or radiolabeled laying hen VLDL (3.1 µg/ml ; specific activity 160 cpm/ng) were incubated with the nitrocellulose in 20 mM Tris-HCl, 90 mM NaCl, 2 mM $CaCl_2$ pH 7.4 for 4 hr. Autoradiography was for 24 hr.

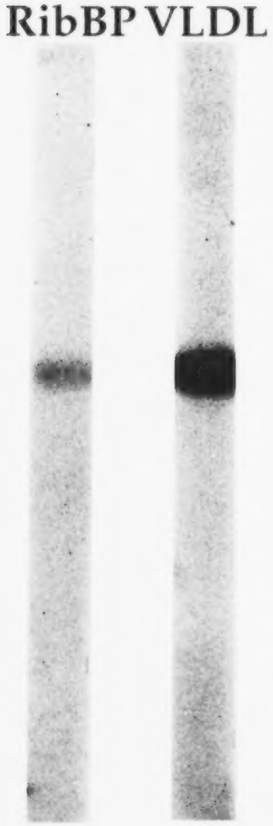
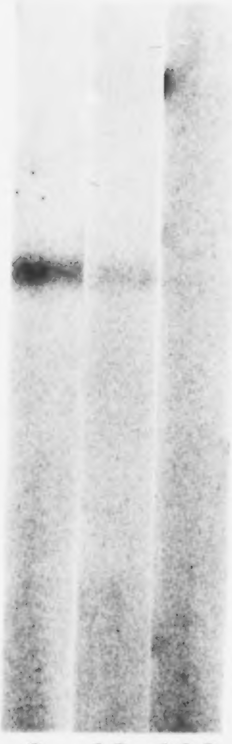

RibBP
VLDL

Figure V. 4.

Inhibition of serum ribBP binding to the 95 kDa oocyte membrane receptor by antireceptor antibody F_{ab} fragments. Oocyte membrane proteins (30 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods (Chapter II). Radiolabeled serum ribBP (1.25 μg/ml; specific activity 8 kcpm/ng) was incubated with the nitrocellulose in 20 mM Tris-HCl, 90 mM NaCl, 2 mM $CaCl_2$ pH 7.4 for 3 hr. Incubations included either 0, 10 or 100 μg/ml anti-LDL receptor F_{ab} fragments. Autoradiography was for 24 hr.



(μg/ml αLDLR Fab)

Figure V. 5.

Inhibition of serum ribBP binding to chicken oocyte membrane receptors by laying hen VLDL. Oocyte membrane proteins (30 µg/lane) were separated by SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods (Chapter II). Radiolabeled serum ribBP (714 ng/ml; specific activity 14 kcpm/ng) was incubated with the nitrocellulose in 20 mM Tris-HCl, 90 mM NaCl, 2 mM $CaCl_2$ pH 7.4 for 5 hr. Incubations included either 0 or 100 fold molar excess laying hen VLDL. Autoradiography was for 50 hr.

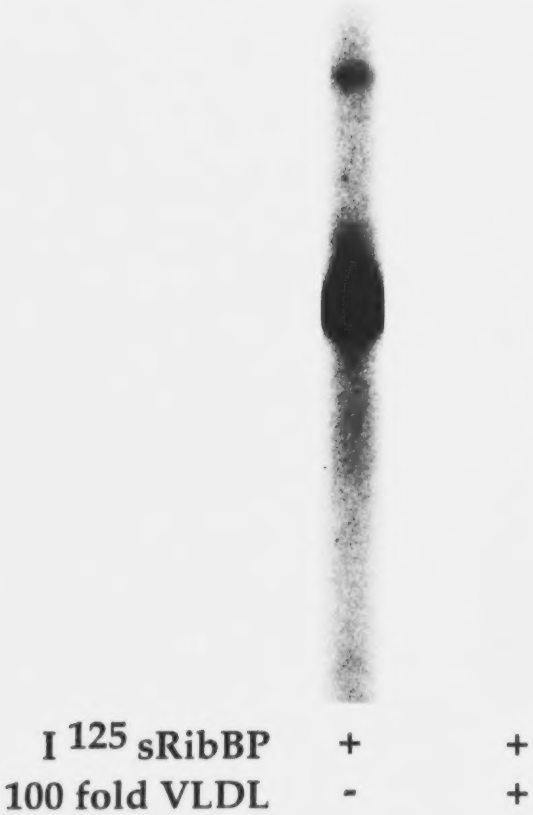

I ^{125} sRibBP + +
100 fold VLDL - +

Figure V. 6.

Radiolabeled laying hen VLDL binding to the 95 kDa oocyte membrane receptor in the presence of excess ribBPs. Oocyte membrane proteins (10 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods (Chapter II). Radiolabeled laying hen VLDL (806 ng/ml; specific activity 620 cpm/ng) was incubated with the nitrocellulose in 20 mM Tris-HCl, 90 mM NaCl, 2 mM $CaCl_2$ pH 7.4 for 4 hr. Incubations included the following additions: lane A, none; lane B, 100 fold molar excess sribBP; lane C, 200 fold molar excess sribBP; lane D, 100 fold molar excess yribBP; lane E, 200 fold molar excess yribBP; lane F, 100 fold molar excess wribBP; lane G, 200 fold molar excess wribBP. Autoradiography was for 24 hr.

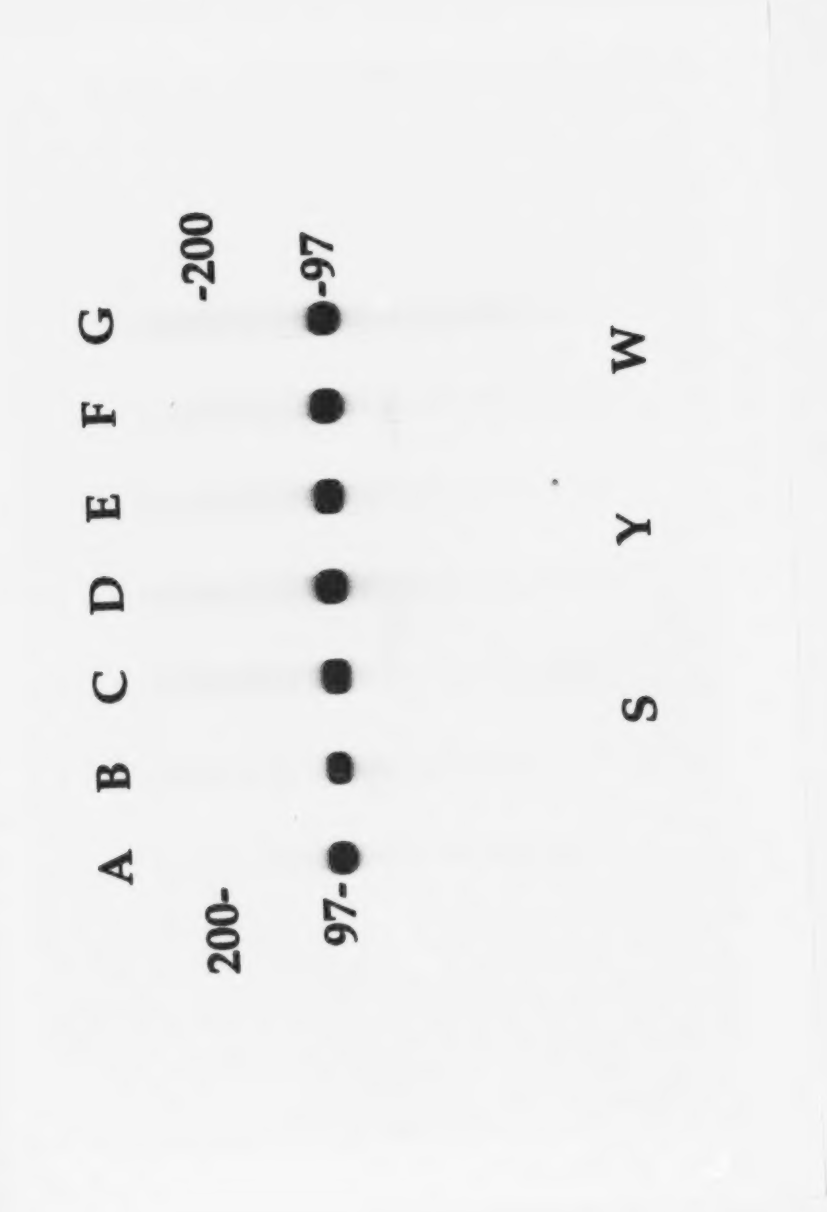

A
B
C
D
E
F
G
200-
97-
-200
-97
S
Y
W

Figure V. 7.

Saturation curve for the binding of ^{125}I-labeled serum ribBP to chicken oocyte membrane octyl-glucoside extracts. Each 100 µl incubation tube contained 18 µg of protein from precipitated octyl-glucoside extract, and the indicated concentrations of ^{125}I-labeled serum ribBP at 2.7 kcpm/ng. Receptor bound ^{125}I-labeled serum ribBP was determined as described in Materials and Methods (Chapter II). Non-specific binding (N.S.) was determined in the presence of 20 mM EDTA. Specific binding was calculated by subtracting non-specific binding (N.S.) from total binding. Each data point represents the average of triplicate determinations.

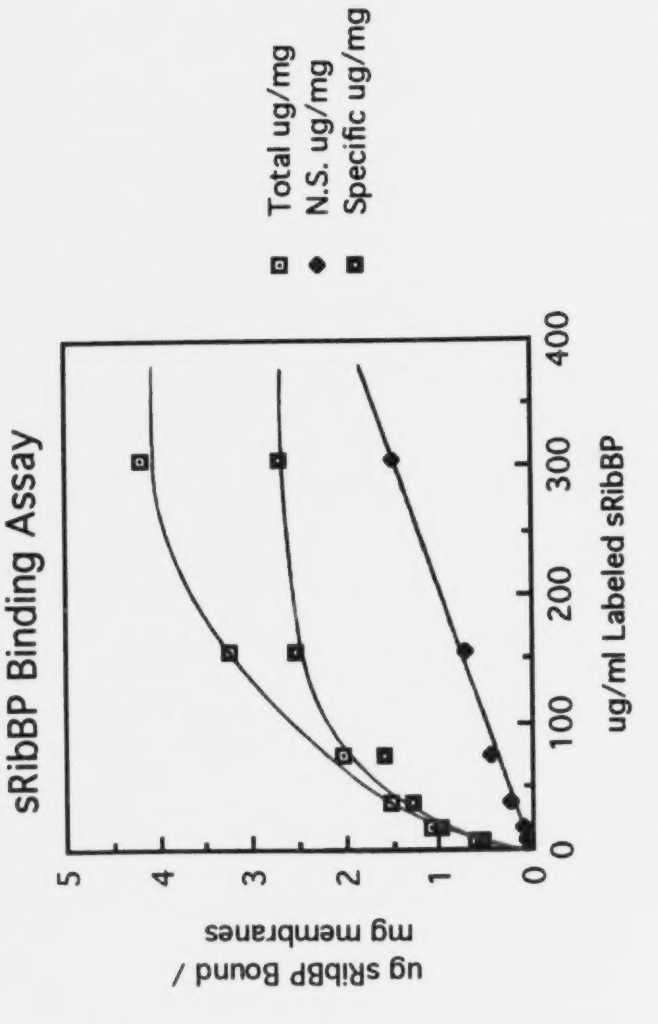

sRibBP Binding Assay
ug sRibBP Bound /
mg membranes
0
1
2
3
4
5
0
100
200
300
400
ug/ml Labeled sRibBP
Total ug/mg
N.S. ug/mg
Specific ug/mg

Figure V. 8.

Scatchard plot for the binding of ^{125}I-labeled serum ribBP to chicken oocyte membrane octyl-glucoside extracts. Specific binding data (Fig. V. 7.) were analyzed by the method of Scatchard. The ratio of bound/free is the amount of receptor bound ^{125}I-labeled serum ribBP (µg) divided by the amount of unbound ^{125}I-labeled serum ribBP (µg) in the reaction mixture.

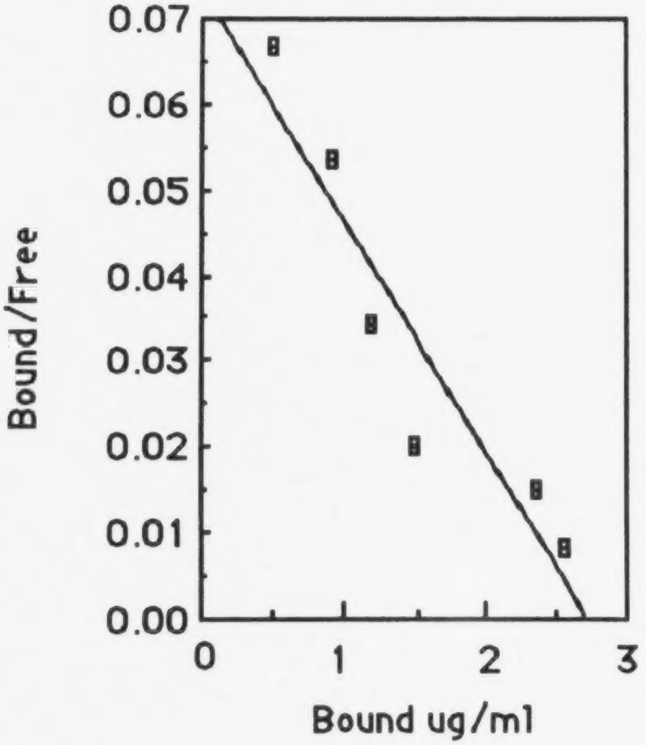

0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
1
2
3
Bound/Free
Bound ug/ml

Figure V. 9.

Competitive binding of ^{125}I-labeled serum ribBP to chicken oocyte membrane octyl-glucoside extracts. Each 100 µl incubation tube contained 18 µg of protein from precipitated octyl-glucoside extract, 18.75 µg/ml of ^{125}I-labeled serum ribBP at 2.7 kcpm/ng and the indicated molar excess of unlabeled serum ribBP. Receptor bound ^{125}I-labeled serum ribBP was determined as described in Materials and Methods (Chapter II). Data is expressed as a percentage of the specific binding observed in the abscence of additional unlabeled serum ribBP. Each data point represents the average of triplicate determinations.

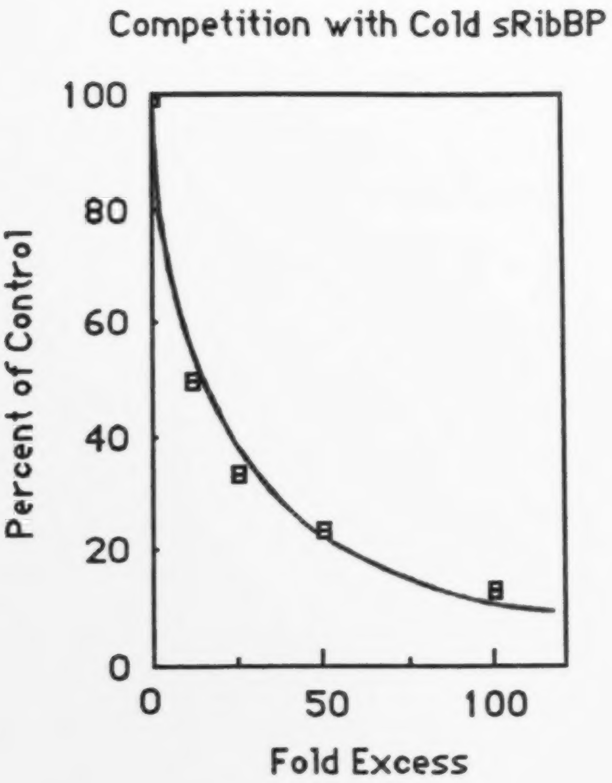
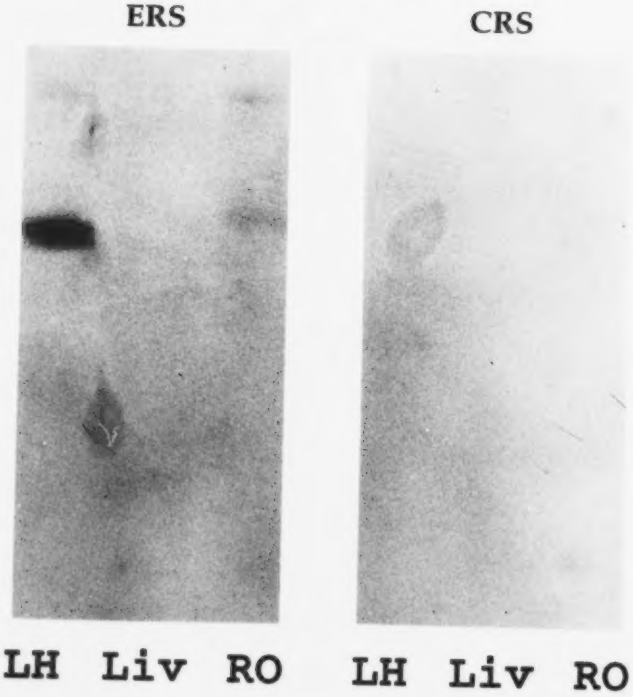

Competition with Cold sRibBP
Percent of Control
100
80
60
40
20
0
0
50
100
Fold Excess

Figure V. 10.

Immunoblot showing the binding of native ribBP in serum to oocyte membrane extracts. Oocyte membrane extracts (20 µg/lane) were transferred to nitrocellulose as described in Materials and Methods (Chapter II). Nitrocellulose panels were incubated with either serum from an estrogen treated rooster, ERS; or control rooster serum, CRS. Following the incubations the bound ribBP was visualized with antibody against egg white ribBP (10 µg/ml) followed by the addition of ^{125}I-labeled Protein A (1.0 µg/ml; specific activity ~ 1 kcpm/ng). Autoradiography was for 18 hr.


ERS
CRS
LH Liv RO
LH Liv RO

CHAPTER VI.

ribBP RECEPTOR BINDING IN ASSOCIATION WITH VTG

VI. ribBP RECEPTOR BINDING IN ASSOCIATION WITH VTG

VI. A. INTRODUCTION

In Chapter V it was shown that ribBP has some affinity for the oocyte membrane lipoprotein receptors and that native ribBP in the presence of other yolk precursors binds strongly to the 95 kDa receptor and to the oocyte LRP. In this chapter the interactions between ribBP and the other yolk precursors are investigated.

Serum ribBP enters the growing follicle with the other yolk precursors via the heavily vascularized theca cell layer which surrounds the oocyte. The yolk precursors move through the theca cell layer and pass between the granulosa cell layer before coming into contact with the oocyte plasma membrane. RibBP and many other yolk precursors are taken up specifically against an increasing concentration gradient. RibBP has several interesting structural features which may play a part in its uptake. RibBP is glycosylated via two N-linked complex oligosaccharides. The composition and structure of the glycosylation varies somewhat between the three forms of the protein (7, 54, 56). The glycosylation of ribBP may have some implications for its uptake (50). Analogous to the cathepsin D mediated cleavage of both VTG and apolipoprotein B (60),ribBP undergoes a specific, carboxy-terminal cleavage upon deposition in the oocyte. (7). A positively charged and hydrophobic sequence (Glu-Lys-Lys-Leu-Leu-Lys) lies near the carboxy-terminus of sribBP at residues 203-208. As discussed in Chapter V this region resembles a region in apo E between residues 143-150 (Lys-Leu-Arg-Lys-Arg-Leu-Leu-Arg) (41), the positively charged region of human apo B between residues 3359-3368 (Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys-Leu) (23)., and the two clusters of positively charged and hydrophobic residues located in the lipovitellin-I portion of VTG at residues 1079-1084 (Lys-Leu-Lys-Arg-Ile-Leu) and 493-498

(Leu-Lys-Arg-Ile-Leu-Lys). These positively charged regions are thought to interact cooperatively with negatively charged SDE repeats on lipoprotein receptors.

The carboxy-terminal portion of the protein contains eight phosphoserines which are thought to play a role in oocytic uptake (51). Dephosphorylation has been found to decrease the rate of oocytic uptake of ^{125}I labelled yolk ribBP *in vivo.* White and Merril (43, 174) have suggested that ribBP could be deposited in the oocyte as part of a complex formed between ribBP and other yolk precursors. They have suggested that ribBP may associate with VTG via calcium phosphate cross-bridges involving the phosphoserines on both proteins. In their model ribBP would be taken up as part of an aggregate "parasitizing" the VTG receptor system.

In order to determine the mechanism of ribBP uptake in the laying hen, I purified ribBP and used it as a ligand in blotting experiments with oocyte membrane extracts. As well, I developed an antibody which recognises ribBP and using this antibody visualized ribBP binding to oocyte membrane extracts. I then determined that this binding is dependent on an interaction with the major yolk precursor VTG. These results are presented here.

VI. B. RESULTS

VI. B. 1. BINDING OF SERUM ribBP TO OOCYTE MEMBRANES

In this chapter I have used ligand blotting techniques to demonstrate binding of native ribBP to oocyte membrane extracts. These results are shown in Fig. VI. 1. The figure shows ribBP in laying hen sera, as visualized with an antibody against ribBP, binding to oocyte membrane proteins with molecular weights of 95 kDa and 380 kDa. These molecular weights correspond to those of the previously characterized 95 kDa receptor for VLDL and VTG and the avian oocyte-specific LDL receptor related protein (LRP) (6) (112) Fig. VI. 1. also shows that there is no signal when serum from a hen homozygous for the *rd* allele is used as a ligand in this type of experiment. As shown in chapter III., the *rd* allele is associated with a point mutation in a 5' splice donor site in the gene for riboflavin binding protein (156). Hens homozygous for this allele do not produce ribBP(44, 69). As such, the *rd* serum provides a convenient genetic control, showing that the signal on the ligand blot is specific for ribBP. This experiment also shows the binding of ribBP in estrogenized rooster serum to these two receptors. However, untreated rooster serum does not contain ribBP and as such there is no signal in the ligand blot when control rooster serum is used as a ligand. In Fig. VI. 2. the 95 kDa and 380 kDa receptors in oocyte membrane extracts are visualized using an antibody which recognizes these two proteins (168). This figure clearly shows the comigration of these receptors and those binding ribBP. The 95 kDa receptor has been shown to bind VTG, apolipoprotein B, α_2 macroglobulin, mammalian apolipoprotein E, and calcium. The oocyte LRP has been shown to bind VTG, α_2 macroglobulin, mammalian apolipoprotein E, calcium and has a low affinity for apolipoprotein B (6, 41, 101, 112). There are no previous reports of either receptor binding ribBP.

VI. B. 2. ASSOCIATION OF ribBP WITH VTG

Chapter V. describes attempts to demonstrate high affinity binding of purified ribBP to either the 95 kDa receptor or the oocyte LRP were unsuccessful. It is possible that in the process of purification some essential cofactor, required for receptor binding and normally present in serum, was removed from ribBP. In particular, one of the other yolk precursors could be involved in the binding of ribBP to these receptors. This hypothesis seemed likely in light of the qualitative differences observed in the ligand binding experiments when using purified vs. native (whole serum) ribBP. In order to test this hypothesis an aliquot of estrogenized rooster serum, rich in ribBP and other yolk precursors, was applied to a gel filtration column and the eluted fractions were analyzed for ribBP as shown in Fig. VI. 3. This analysis demonstrated that under the conditions used in the ligand blotting experiments, ribBP eluted from the Superose 6 column in a fraction containing proteins of substantially higher molecular weight. When the sample was applied in buffer which contained 20 mM EDTA the majority of ribBP eluted in the same fraction as pure ribBP. These results would indicate a cation-dependant interaction between ribBP and one or more other serum proteins. In order to characterize this phenomenon an aliquot of estrogenised rooster serum was chemically cross-linked using the cross-linking reagent 1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride. The cross linked serum was then subjected to SDS PAGE, transferred to nitrocellulose and probed with an affinity purified antibody against ribBP (Fig. VI. 4). This experiment demonstrated a significant and specific shift in electrophoretic mobility of ribBP when crosslinked in whole sera. White and Merrill proposed that the oocytic uptake of ribBP may involve aggregation with VTG which is in turn taken up by the VTG receptors (43). The shift in mobility from an apparent molecular weight of 39 kDa to an apparent molecular weight of 280 kDa was consistent with cross linking specifically to VTG. The nitrocellulose was then stripped of antibody and protein A-peroxidase by treatment with a solution containing β-mercaptoethanol and SDS as described in Materials and Methods (Chapter II). When reprobed with an antibody directed against VTG, a band with identical electrophoretic mobility to that observed with the antibody against ribBP could be visualized. This is consistent with a model which would include an association between ribBP and VTG in serum.

VI. B. 3. RECEPTOR BINDING OF VTG ASSOCIATED ribBP

Given the apparent association between ribBP and VTG in serum, ligand blotting experiments were carried out using ^{125}I labeled FPLC purified serum ribBP as a ligand. The labeled ribBP was supplemented with equimolar amounts of native VTG. Some of the incubations also included 1 mM Ca^{2+} and/or 1 mM P_i. The inclusion of 1 mM Ca^{2+} was rationalized by the apparent requirement for a divalent cation in the association between ribBP and VTG (Fig. VI. 3). The inclusion of 1 mM P_i in the incubation was rationalized in part by the recent report that ribBP could self associate in the presence of Ca^{2+} and P_i (52). As well, ribBP has been compared to casein proteins which contain similarly phosphorylated regions (52). Caseins are known to associate with other serum proteins in a manner which is dependant on Ca^{2+} and P_i. The results of these ligand blotting experiments are shown in Fig. VI. 5. Firstly, under these conditions and exposure times there is no observable binding of pure ribBP alone to the 95 kDa or the LRP. The addition of 1 mM Ca^{2+} and/or 1 mM P_i had no result. However upon the addition of an equimolar amount of VTG a signal is visible in the ligand blot. This effect is maximalized upon the addition of both 1 mM Ca^{2+} and 1 mM P_i to the incubation. While 1 mM Ca^{2+} alone has an enhancement effect in the presence of VTG, 1 mM P_i apparently does not. Although the molecular weight of the band visualized in these ligand blots corresponds to the 95 kDa oocyte membrane receptor more direct evidence for binding to this protein is shown in Fig. VI. 6. This figure shows that the binding of labeled ribBP to oocyte membranes in the presence of VTG, 1 mM Ca^{2+} and 1 mM P_i can be competed by F_{ab} fragments from rabbit IgG which recognizes the 95 kDa and 380 kDa receptors. Given these results an experiment was performed to test whether native ribBP could compete for the interaction between ^{125}I labeled ribBP, VTG and the 95 kDa oocyte membrane receptor. The purpose of this experiment was two-fold. Firstly it was important to demonstrate some competitive ability in order to preclude an important artifact. This artifact could result from free ^{125}I associating with the VTG in solution which is known to bind to the receptors in question. However if the signal in the ligand blots can be competed by native ribBP this would demonstrate that the signal on the ligand blot is the result of a protein-protein interaction. Indeed this is the case since the signal resulting from ^{125}I labeled serum ribBP binding to the 95 kDa receptor in the presence of equimolar VTG , 1 mM Ca^{2+} and 1 mM P_i can be competed by the addition of excess native serum ribBP to the incubation (Fig. VI. 7). Both serum and egg white ribBP have the same amino acid sequence yet differ in the composition of their post translational glycosylation. Fig. VI. 7 shows that egg white ribBP can compete for receptor binding in spite of these differences. This implies that the carbohydrate composition of ribBP is not important for receptor binding. Similarly, the egg yolk ribBP differs from serum ribBP in the removal of the carboxyterminal 11 or 13 amino acids. Fig. VI. 7 shows that in spite of the loss of the carboxyterminus, egg yolk ribBP competes with serum ribBP for receptor binding. This implies that the carboxyl terminal 11 or 13 residues do not play a role in receptor binding. In order to further demonstrate that the carboxyterminal 11 residues of serum ribBP are not directly involved in receptor binding we attempted to compete for the binding of radiolabeled serum ribBP with an excess of peptide which corresponded to these residues (Fig. VI. 8). The carboxyterminal peptide Phe-Glu-Ala-Leu-Gln-Gln-Glu-Glu-Gly-Glu-Glu was unable to compete with serum ribBP for the binding to the 95 kDa receptor in the presence of equimolar VTG , 1 mM Ca^{2+} and 1 mM P_i. This again indicates that the carboxyterminal terminal portion of serum ribBP is not involved in receptor binding or in the interaction with VTG.

VI. C. DISCUSSION

Previous attempts to delineate the mechanism of ribBP uptake had failed to demonstrate high affinity receptor binding. However, the accumulation of ribBP in the yolk, against the concentration gradient, suggests a specific receptor mediated process. In these experiments I have demonstrated binding of ribBP to a known oocyte membrane receptor, the 95 kDa oocyte membrane receptor for VLDL and VTG. I have also demonstrated binding to the oocyte LRP. Fig. VI. 7. shows the binding of purified serum, egg yolk and egg white ribBPs to the oocyte LRP. Binding to the LRP is not shown in Figs. 5, 6 and 8. This is most likely due to the variable quality of the electrophoretic transfer of higher molecular weight membrane proteins. This variability has been observed previously in other experiments with ligands for these receptors(1, 2, 6, 168). Binding to LRP was observed upon longer exposure of the autoradiographs in these experiments (data not shown). Binding of ribBP to the 95 kDa receptor involves an association with VTG and requires Ca^{2+} and P_i. White and Merrill proposed that ribBP may be deposited in the oocyte by a mechanism which parallels the one elucidated here (43). They proposed that ribBP forms aggregates with VTG and is taken up by the VTG receptors. Recently a self associating behavior for ribBP has been demonstrated (52). RibBP was shown to dimerize in the presence of Ca^{2+} and P_i. This dimerization is thought to involve interactions with the phosphoserine residues in ribBP and calcium phosphate. These interactions are similar to those of the caseins and calcium phosphate. Caseins are phosphoproteins which associate with calcium phosphate to form colloidal particles called casein micelles (175). The calcium phosphate associated with such micelles is called micellar calcium phosphate. Given the previously demonstrated self association behavior of ribBP and the heavily phosphorylated nature of VTG, it seemed likely that ribBP and vitellogenin could form hetero-complexes via similar mechanisms. In this study I demonstrate that ribBP and vitellogenin are capable of complex formation. Furthermore I demonstrate that the amounts of calcium and phosphate required for this interaction are within the range of the concentrations found in vivo. Finally we demonstrate that under these same conditions radiolabeled ribBP is capable of binding to the 95 kDa receptor in concert with VTG.

Various studies have attempted to determine which of the properties of ribBP are important for its uptake into the oocyte. Dietary studies have demonstrated that riboflavin is not required for the deposition of ribBP in the oocyte. The concentration of ribBP in the serum, yolk and albumin is independent of dietary riboflavin (65). Therefor any receptor mediated uptake mechanism must be incapable of distinguishing between the apo and holo forms of riboflavin binding protein. Although there are some structural changes in ribBP associated with binding of riboflavin, these must not be in themselves enough to disrupt the uptake mechanism (63). This is important in light of the fact that egg white ribBP is capable of binding to the 95 kDa oocyte membrane receptor and can compete effectively with serum ribBP for receptor binding. Although egg white ribBP is synthesized in the oviduct and serum ribBP is synthesized in the liver, the two proteins share the same amino acid sequence and differ in only two respects. Firstly, the majority of egg white ribBP remains in the apo form whereas essentially all of the serum form has bound riboflavin associated with it (43). Secondly, the composition of the carbohydrates attached to the two forms is different. Both forms have approximately 12 N-acetylglucosamine residues and 6 mannose residues. As well, the serum form of ribBP has approximately five sialic acid, seven galactic acid residues and one fucose residue while egg white ribBP has one sialic acid residue, two galactose residues and lacks fucose(7). Previous uptake studies showed that following intravenous injection of radiolabeled ribBPs in laying hens egg white ribBP was taken up in the oocytes more slowly than was serum ribBP. However, the authors also demonstrated that egg white ribBP was cleared by the liver much faster than serum ribBP(50). The authors concluded from their study that the difference in oocytic uptake was directly related to the increased hepatic uptake of egg white ribBP. My results would support their conclusions. I show that egg white ribBP is able to compete effectively with serum ribBP for binding to the 95 kDa receptor in concert with vitellogenin. This indicates that a role for the N-linked oligosaccharides present on the serum form of the protein in the interaction with either VTG or the 95 kDa receptor is unlikely.

The egg yolk form of ribBP differs from the serum form in two respects. Following deposition in the oocyte the ribBP undergoes a specific carboxyterminal cleavage of 11 or 13 residues. This cleavage is analogous to the cleavage of both VTG and apolipoprotein B in VLDL. Yolk ribBP also differs from serum ribBP due to the hydrolysis of one sialic acid, one fucose, two galactose and three N-acetylglucosamine residues following oocytic uptake(54).My results show that yolk ribBP is also able to compete with serum ribBP for receptor binding. This ability to compete for receptor binding must take into account one or more of three factors. Firstly the differences in glycosylation do not result in an appreciable change in binding affinity. Secondly the carboxyterminal 11 or 13 residues on serum ribBP must not be directly involved in receptor binding either via interaction with the 95 kDa receptor itself or via interaction with VTG or a combination of these two. Thirdly, any conformational change induced by the removal of the carboxyterminal 11 or 13 amino acids in serum ribBP must be so slight as to not interfere with the interactions required for binding to the 95 kDa receptor. One could envision a model whereby the interaction between ribBP and vitellogenin involves micellar calcium phosphate bridges between the phosphoserines in ribBP and VTG. In ribBP these phosphoserines are clustered close to the carboxyterminus. Removal of the carboxyterminal 11 or 13 residues could result in a conformational change which makes the phosphoserines much less accessible to VTG thereby disrupting the interaction between the two proteins and in turn inhibiting binding to the receptor. However this appears to not be the case. In order to further demonstrate that the carboxyterminal 11 residues of serum ribBP are not directly involved in the binding to the receptor I attempted to compete for the receptor binding of radiolabeled serum ribBP with an excess of peptide which corresponded to these residues (Fig. VI. 8). The carboxyterminal peptide was unable to compete with serum ribBP for the binding to the receptor. This again clearly indicates that the carboxyterminal terminal portion of serum ribBP is not involved in receptor binding or in the interaction with VTG.

My results indicate that the oocytic uptake of riboflavin binding protein could be mediated by an interaction with vitellogenin. This interaction facilitates binding to both the 95 kDa oocyte membrane receptor and the oocyte LRP. One could expect that such a mechanism could be conserved through evolution. Riboflavin binding proteins are found in oviparous species as diverse as fish, reptiles, amphibians and birds. In particular riboflavin binding proteins have been characterized in carp (*Cyprinus carpio*) (70), alligator (*Alligator mississippiensis*) (71), Indian python (*Python molurus*) (72), painted turtle (*Chrysemys picta*) (72), Japanese quail (*Coturnix japonica*) (73), muscovy duck (*Cairina moschata*) (72) and of course the chicken. The riboflavin binding proteins in reptiles and amphibians have similar amino acid compositions and molecular weights to their avian counterparts (72). As well, they all appear to be phosphoglycoproteins. It is likely that they are all coded for by related genes. One could assume that the riboflavin binding proteins in oviparous species could have a conserved function as well, namely to facilitate the transport of riboflavin into the growing oocyte. This appears to be the case since all of these proteins are found, associated with riboflavin, in the eggs of these animals. The uptake of vitellogenins in diverse species has been a subject of inquiry in our laboratory. Specifically Stefani et al. have characterized piscine and amphibian counterparts to the avian 95 kDa receptors (40) (176). These receptors bind the vitellogenins from these species and show remarkable cross reactivity in that they bind avian vitellogenin as well. This is indicative of the intense evolutionary pressure to conserve the mechanism of vitellogenin uptake. Given the high degree of conservation on this level, it would seem appropriate to assume that the involvement of riboflavin binding proteins in this mechanism may be conserved as well. The oocytic uptake of riboflavin binding proteins via interaction with vitellogenin and the vitellogenin receptors may be a common theme through evolution.

Figure VI. 1.

Oocyte receptor binding of serum-borne ribBP. Oocyte membrane extracts (10 µg/lane) were transferred to nitrocellulose as described in Materials and Methods (Chapter II). Nitrocellulose strips were incubated with: laying hen serum, LHS; serum from a hen homozygous for the *rd* allele, rdS; serum from estrogen-treated roosters, ERS; or control rooster serum, CRS. Following the incubations, the bound ribBP was visualized with affinity-purified antibody against serum ribBP (0.26 µg/ml). Exposure time was 30 sec. Migration positions of the 200-kDa and 97-kDa protein standards are indicated.

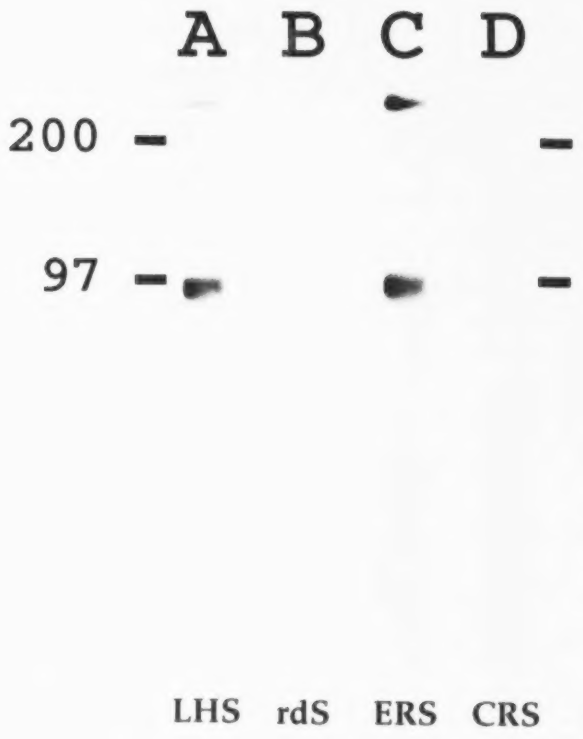

A
B
C
D
200
97
LHS
rdS
ERS
CRS

Figure VI. 2.

Immunoblotting of the oocyte-specific members of the low density lipoprotein receptor family. Oocyte membrane extracts (10 μg/lane) were transferred to nitrocellulose as described in Materials and Methods (Chapter II). (A), the nitrocellulose strip was incubated with antibodies which recognize both the 95-kDa oocyte receptor and the oocyte LRP (0.25 μg/ml). (B), the nitrocellulose strip was incubated in serum from an estrogen-treated rooster as in Fig. VI. 1, and bound ribBP was visualized with affinity-purified IgG against serum ribBP (0.52 μg/ml). Visualization was by ECL; exposure time was 15 sec. Migration positions of the 200-kDa and 97-kDa protein standards are indicated.

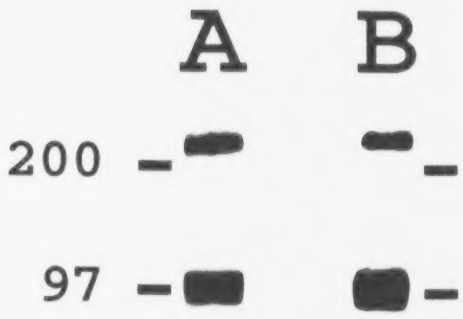

A
B
200
97

Figure VI. 3.

Gel chromatographic and immunoblotting analysis of chicken serum ribBP. Elution profile of serum from an estrogen-treated rooster on a Superose 6 HR 10/30 column. Serum was loaded onto a Superose 6 column and the ribBP in the eluted fractions was visualized by immunoblotting with affinity-purified antibody against serum ribBP (0.26 µg/ml) as described in Materials and Methods (Chapter II). Exposure time was 5 min. The column was calibrated with 50 µg of pure serum ribBP which eluted in the fraction indicated by the *arrowhead.*

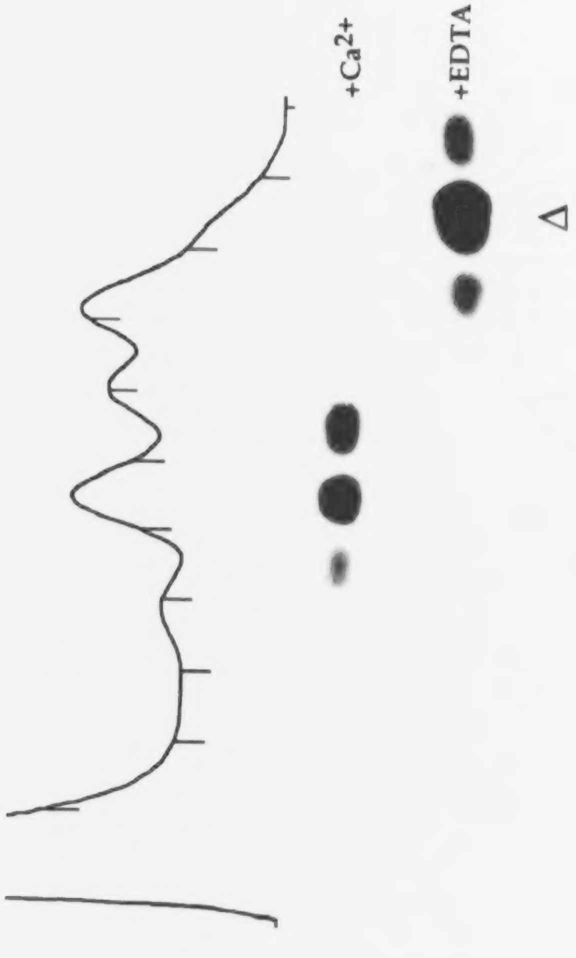

+Ca2+
+EDTA
Δ

Figure VI. 4.

Chemical crosslinking of ribBP in serum. The proteins in the serum of an estrogen-treated rooster were chemically crosslinked as described in Materials and Methods (Chapter II). Native (A) and crosslinked (B) sera were subjected to SDS-PAGE and visualized with affinity-purified IgG directed against ribBP (0.26 μg/ml). Autoradiography in panels A and B was for 60 min. After stripping (see Materials and Methods), the nitrocellulose of lane B was reprobed with antibody directed against VTG (0.75 μg/ml) (D). Panel C shows the migration position of vitellogenin in native serum visualized with the same antibody. Autoradiography in panels C and D was for 25 sec. The migration positions of molecular weight markers (kDa) are indicated on the left hand side.

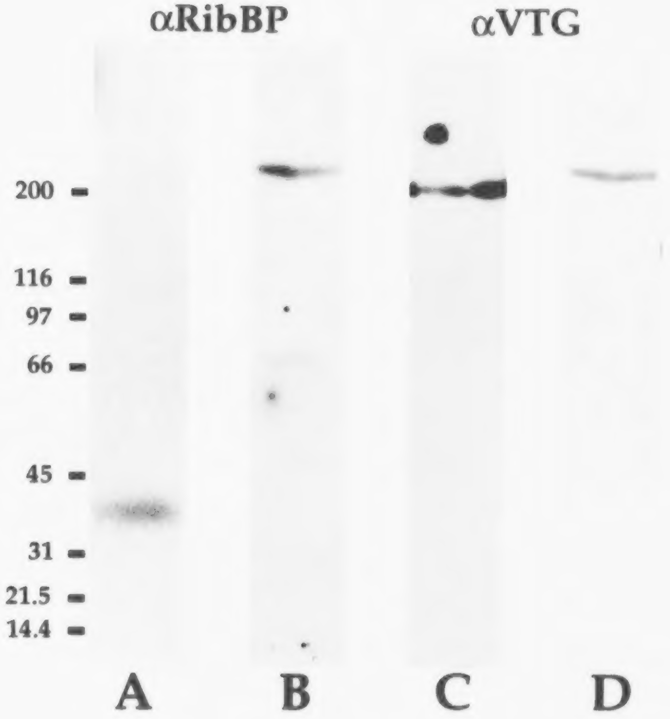
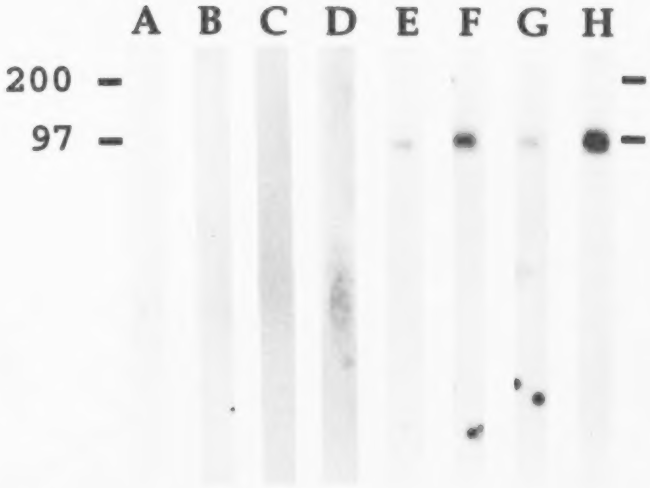

αRibBP
αVTG
200
116
97
66
45
31
21.5
14.4
A
B
C
D

Figure VI. 5.

Ligand blotting using radiolabeled pure serum ribBP. Oocyte membrane proteins (10 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods (Chapter II). Radiolabeled serum ribBP (65 ng/ml; specific activity 15 kcpm/ng) was incubated with the nitrocellulose in buffers which did or did not contain 1 mM Ca^{2+}, 1 mM P_i (PO_4^{3-}), or VTG (1.4 μg/ml) as indicated. Autoradiography was for 25 hr. Migration positions of the 200-kDa and 97-kDa protein standards are indicated.


A
B
C
D
E
F
G
H
200
97

	A	B	C	D	E	F	G	H
VTG	-	-	-	-	+	+	+	+
Ca^{2+}	-	+	-	+	-	+	-	+
PO_4^{3-}	-	-	+	+	-	-	+	+

Figure VI. 6.

Inhibition of ribBP binding by anti-oocyte receptor F_{ab} fragments. F_{ab} fragments were prepared from rabbit anti-95-kDa IgG. Oocyte membrane proteins (10 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with radiolabeled serum ribBP (74 ng/ml; specific activity 13.5 kcpm/ng) in buffers which contained 1 mM Ca^{2+}, 1 mM P_i, and VTG (1.4 μg/ml) with the following additions: A, none; B, 250 μg/ml F_{ab} fragments; C, 500 μg/ml F_{ab} fragments. Autoradiography was for 18 hr. Migration positions of the 200-kDa and 97-kDa protein standards are indicated.

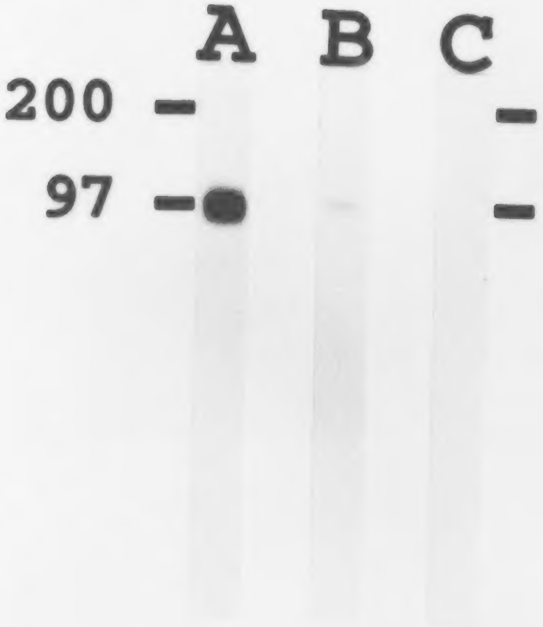

A
B
C
200
97

Figure VI. 7.

Receptor binding of the different forms of ribBP. Oocyte membrane proteins (10 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods (Chapter II). Nitrocellulose was incubated in buffers containing 1 mM Ca^{2+}, 1 mM P_i , and VTG (1.4 μg/ml) with the following additions: lane A, radiolabeled serum ribBP (74 ng/ml; specific activity 13.5 kcpm/ng); lane B, radiolabeled egg yolk ribBP (64 ng/ml; specific activity 15.6 kcpm/ng); lane C, radiolabelled egg white ribBP (69 ng/ml; specific activity 14.5 kcpm/ng); Lanes D, E and F, radiolabeled serum ribBP (74 ng/ml; specific activity 13.5 kcpm/ng) with the following additions of unlabeled ribBPs (each at 37 μg/ml, representing a 500-fold molar excess): lane D, serum ribBP; lane E, egg yolk ribBP ; and lane F, egg white ribBP. Autoradiography was for 60 hr. Migration positions of the 200-kDa and 97-kDa protein standards are indicated.

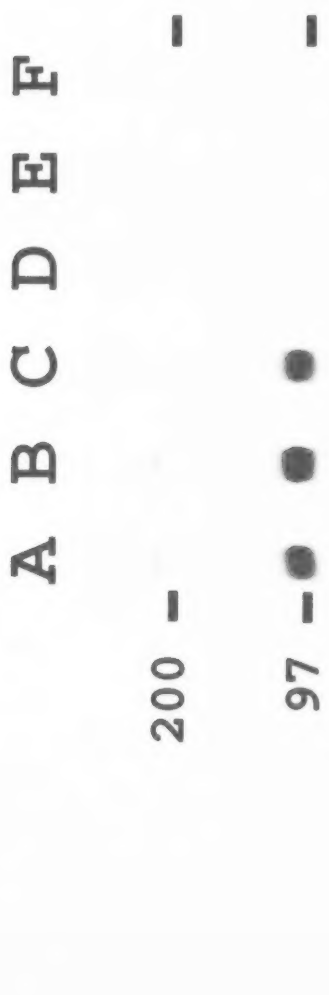
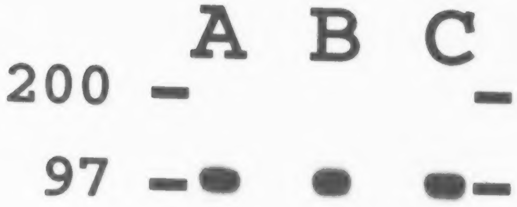

A
B
C
D
E
F
200
97

Figure VI. 8.

The carboxyterminal peptide of sribBP is not involved in receptor binding. Oocyte membrane proteins (10 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods (Chapter II). The nitrocellulose strips were incubated with radiolabeled serum ribBP (74 ng/ml; specific activity 13.5 kcpm/ng; ~ 2.2×10^{-12} moles) in buffers which contained 1 mM Ca^{2+}, 1 mM P_i, and VTG (1.4 μg/ml) with the following additions: A, none; B, 2.2×10^{-9} moles carboxyterminal peptide (1000-fold molar excess); and C, 1.1×10^{-8} moles peptide (5000-fold molar excess). The peptide corresponded to the 11 residues that are removed from ribBP following oocytic uptake and had the sequence: Phe-Glu-Ala-Leu-Gln-Gln-Glu-Glu-Gly-Glu-Glu. Autoradiography was for 18 hr. Migration positions of the 200-kDa and 97-kDa protein standards are indicated.


A
B
C
200
97

CHAPTER VII.

GENERAL DISCUSSION

The laid egg contains a delicate balance of nutrients destined to fuel the growth and development of the embryo. The two major yolk precursors, VLDL and vitellogenin (VTG), provide the embryo with sources of triglycerides, phospholipids, cholesterol, amino acids, calcium and phosphate. Their uptake is mediated by the 95 kDa oocyte plasma membrane receptor, which specifically binds and internalizes both of these components (6). Riboflavin binding protein is one of a group of minor yolk precursors, livetins, which constitute approximately 10% of the total dry weight of the yolk. Other livetins include retinol binding protein, biotin binding protein, transferrin, immunoglobulin Y and α_2-macroglobulin (42). Although the minor yolk precursors are equally requisite for embryonic development, their uptake mechanisms for the most part remain to be determined.

In 1988, when this study was begun, very little was known about the oocytic uptake of ribBP. At that time it seemed likely that there may a specific, novel, receptor for ribBP. The ligand blotting experiments in Chapter V are the first to demonstrate that sribBP binds to the 95 kDa oocyte membrane receptor and the oocyte LRP. This binding is specific and can be competed with excess unlabeled sribBP. The binding of sribBP to the 95 kDa receptor is analogous to apoB mediated VLDL binding to the same receptor. Both sribBP and VLDL binding require calcium (1). Both interactions are abolished by the heterocyclic, polyanionic compound suramin (1).Serum ribBP binding to the 95 kDa receptor can be competed by VLDL. As well, antibodies which abolish VLDL binding to the 95 kDa receptor also abolish the binding of sribBP.

The 95 kDa receptor has been shown to bind apoB containing lipoproteins, VTG, Ca^{2+}, α_2-macroglobulin and the mammalian ligand apolipoprotein E (41). The binding of these various ligands to the 95 kDa receptor is thought to be mediated by the type A ligand binding or SDE repeats. These negatively charged SDE repeats are thought to interact cooperatively with positively charged regions on the ligands. A positively charged region of human apo B has been implicated in binding to the LDL receptor (23).This region is located between residues 3359-3368 (Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys-Leu). Vitellogenin contains two clusters of positively charged and hydrophobic residues located in the lipovitellin-I position of VTG at residues 1079-1084 (Lys-Leu-Lys-Arg-Ile-Leu) and 493-498 (Leu-Lys-Arg-Ile-Leu-Lys). These regions resemble a region in the putative receptor binding site of apo E between residues 143-150 (Lys-Leu-Arg-Lys-Arg-Leu-Leu-Arg) (41). A positively charged and hydrophobic sequence (Glu-Lys-Lys-Leu-Leu-Lys) lies near the carboxy-terminus of sribBP at residues 203-208. These residues may mediate the binding of ribBP to the avian lipoprotein receptor family. Interestingly, the post-endocytotic processing of yribBP involves cleavage at either residue 206 or 208. In the later case this would remove two of the six residues in this putative receptor binding region.

The results of the quantitative binding assay indicate that the binding of ribBP to oocyte membrane proteins is saturable, that there is a finite number of binding sites. However this assay also yields a very high K_d for ribBP binding to oocyte membranes (1.3 μM), therefor the affinity of ribBP for oocyte membrane proteins is low. Since the affinity of pure ribBP for oocyte membranes is not of the magnitude required to facilitate oocytic uptake I investigated the possibility that ribBP associated with other yolk precursors. In Chapter VI I report that in serum, ribBP associates with VTG, a ligand of the 95-kDa receptor. In the presence of VTG, ^{125}I-labeled sribBP binds to the 95-kDa receptor, and under certain conditions also to the avian oocyte low density lipoprotein receptor-related protein. The interaction between ribBP and the 95-kDa receptor and/or VTG requires Ca^{2+} and P_i.

There are three forms of ribBP in the laying hen. Synthesized in the liver under the control of estrogen, sribBP enters the serum and is delivered to yolk where it becomes carboxyterminally truncated (yribBP); the egg white form (wribBP), synthesized by the oviduct, is a product of the same gene as sribBP, but has a different glycosylation pattern. Interestingly, sribBP, yribBP and wribBP are all capable of VTG-associated receptor binding. This demonstrates that (i) the carboxyterminal 11 or 13 amino acids which are removed from sribBP upon oocytic uptake are not involved in receptor binding, and (ii) receptor binding and/or association of ribBP with VTG is not dependent on the carbohydrate structure present on sribBP. These results indicate that the oocytic uptake of sribBP is mediated, through association with VTG, by the 95-kDa receptor and possibly the other oocytic member of the low density lipoprotein receptor gene family, the oocytic LRP. This adds an interesting and novel variation to ligand recognition by these receptors.

In spite of the considerable body of evidence presented in this thesis to support the idea that the uptake of ribBP is mediated by oocytic members of the low density lipoprotein receptor gene family, some of this evidence relies on certain presumptions which should be made clear. For example, the methods used to prepare and analyze oocyte membrane extracts are in themselves potentially disruptive to any receptor activity. During membrane preparation samples are sonicated and subjected to electrophoresis in the presence of SDS. Such treatment is capable of denaturing secondary and tertiary protein structure. The methodology used in this thesis assumes that any receptor present in oocyte membrane extracts is capable of reassuming its active conformation following electrophoretic transfer. Members of the low density lipoprotein receptor family are capable of renaturing in this way, but perhaps other receptors are not. For example receptors composed of heterocomplexes associating non-covalently would be expected to disassociate following such treatment and potentially lose receptor binding activity. Therefor the methods used in the course of this study have the potential to prejudice the results such that receptors which share the characteristics of the low density lipoprotein receptor family will be detected preferentially to those which do not. Other types of experiments which could be used to confirm the results presented in this thesis include immunoprecipitation of receptor ligand complexes, cross linking of receptor ligand complexes and cellular uptake studies using cells which have been transfected with expression constructs coding for the oocytic members of the lipoprotein receptor family. The first two methods have the potential to identify other proteins in oocyte membranes which might have ribBP binding activity. All of these methods could potentially verify the ligand binding results presented in this thesis.

I have investigated the mechanisms underlying riboflavin transport pathways by molecular characterization of a relevant mutation in chicken. The autosomal recessive *rd* allele in *Gallus gallus domesticus* prevents the synthesis of functional ribBP and induces embryonic death on day 13. Intrigued by the possibility that the disease riboflavinuria may be caused by a defect in the ligand ribBP which effected its putative receptor mediated uptake, I chose to determine the nature of the *rd* allele. PCR was used to amplify and clone cDNAs for ribBP from normal (*Rd)* and deficient (*rd*) animals. The *rd* allele was associated with a 100-nucleotide deletion in the messenger RNA for ribBP. Genomic clones were generated via PCR using primers flanking this 100 bp deletion, and the resulting 2.1 kb clones were sequenced. The deletion in the *rd* ribBP cDNA corresponds precisely to an exon. The splice site following this exon contains a G to A mutation at position 1 of the downstream 5′ splice donor sequence. The effect of this anomaly and the cause of the *rd* phenotype is the disruption of the normal splicing pathway. Four rare splicing products were identified which are results of the *rd* mutation in the gene for riboflavin binding protein. The most abundant of these splicing products, Δ100, is the product formed by skipping of a 100 bp exon in the gene for ribBP. A second product, Δ44, is the result of cryptic splice site usage. A cryptic 5' splice donor site, containing the sequence GTAAGC, lies in the coding region of the gene for riboflavin binding protein . This site is used when the wild type 5' splice donor site is perturbed due to the G to A mutation in the *rd*. . The use of this splice-site and subsequent formation of the Δ44 splicing product could be predicted based on its sequence complementarity to the U1 snRNA. A third splicing product, Δ231, results from skipping of not one but two exons upstream from the *rd* mutation. A fourth and entirely unexpected product, Δ0, results from maintenance of the normal splicing pattern in spite of the G to A mutation. Although four splicing products were characterized, the majority of the *rd* transcript would be expected to be trapped in a dead end lariat conformation. For this reason ribBP message is not observed upon Northern analysis of *rd* transcripts and as such there is no ribBP produced in hens homozygous for the *rd* allele.

The research presented in this thesis had the following objectives: i.) to elucidate the mechanism by which ribBP is taken up into the growing oocyte and ii) to delineate the molecular basis of the inherited disease avian riboflavinuria. With respect to the first objective, my results indicate that the oocytic uptake of sribBP may be mediated, through association with VTG, by the 95-kDa receptor and possibly other oocytic members of the low density lipoprotein receptor gene family. Secondly, I have shown that avian riboflavinuria, as characterized by a lack of riboflavin binding protein, is caused by a G to A mutation in the non-coding region of the gene for riboflavin binding protein. As a result of this mutation there is no ribBP found in the serum, egg yolk or egg white of hens homozygous for the *rd* allele.

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